The Biochemical Journal

Volume 98, 1966

The Biochemical Journal

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London: The Biochemical Society

Published by
THE BIOCHEMICAL SOCIETY, 20 PARK CRESCENT, LONDON, W.1
and distributed for the Society by
THE CHEMICAL SOCIETY, BURLINGTON HOUSE, PICCADILLY,
LONDON, W.1

Printed in Great Britain by Spottiswoode, Ballantyne & Co. Ltd.,
London and Colchester

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THE BIOCHEMICAL JOURNAL

POLICY OF THE JOURNAL AND INSTRUCTIONS TO AUTHORS

Papers in English are accepted from any country whether or not the author is a member of The Biochemical Society. It is the policy of the *Biochemical Journal* to publish papers in all fields of biochemistry, provided that they describe results which make a sufficient contribution, either experimentally or theoretically, to knowledge, or that they describe new methods applicable to biochemical problems. This statement of policy has been approved by the Committee of the Society. Its interpretation is in the hands of the Editorial Board, who judge whether each paper submitted falls within the limits prescribed. Clearly it is impossible to devise any hard and fast working rule for judging the acceptability of papers on grounds of subject-matter, and occasional inconsistency of treatment is bound to occur.

Length of papers. No upper limit is set to the length of a paper, provided that this is commensurate with its scientific content. Likewise, no lower limit is set; *a paper may be very short, provided that it makes some valid biochemical statement and supports this by adequate experimental detail.* The acceptance of papers is in no way dependent on the total number of papers being considered for publication. If a paper is scientifically acceptable room will always be found for it in the Journal.

Assessment of papers. Papers are assessed with regard to the following questions:

(1) whether the subject-matter falls within the terms of reference;

(2) whether there is a sound basis for the conclusions drawn;

(3) whether the experimental methods are described clearly and fully enough to allow the work to be exactly repeated;

(4) whether the results and discussion are clearly set out;

(5) whether the presentation is reasonably concise.

Functions of the Editorial Board. Members of the Editorial Board are appointed by the Committee of the Society on the recommendation of the Editorial Board. The aim is to have a Board whose members have a wide knowledge of biochemistry and in which fields of expert knowledge are so distributed that most papers can be dealt with.

Normally a paper is read by at least two members of the Editorial Board. If an expert opinion in the subject is not available among members of the Board, the paper is submitted to a referee. The main task of Editors and referees is to assess the paper under heads (1) to (5), and to make recommendations on the acceptability of the paper, subject to any revision that may be required. Recommendations for revision are normally in the form of one or more Editorial Reports. After it has been revised by the author, the paper is seen again by at least one Editor, to check whether it is now ready for preparation for the press by the Editorial Office. In preparation for the press, particular attention is paid to grammar and the conventions of the Journal with regard to nomenclature, symbols, illustrations, Tables and References. (Standardization, within reason, and precision of terms and phrasing are helpful, especially to foreign readers.) If rejection of a paper is recommended, or if there is any serious disagreement between Editors, the paper is read by the Chairman or his Deputy.

The Secretary to the Editorial Board, who is in charge of the Editorial Office, administers the business of the Journal, in consultation with members of the Board and with the Chairman of the Board, to whom he is responsible.

The Editorial Board meet twice a year to discuss matters related to the production of the Journal. Subcommittees, which report to the Board, are appointed to consider such matters as nomenclature and abbreviations. The Board report to the Committee of the Society, whose decision is required on financial aspects, appointments and major aspects of policy.

Relations between authors and the Editorial Board. The aim of the Editorial Board is to maintain a high standard both of subject-matter and of its presentation. Requests for revision range from

uncontroversial matters, such as the correct citing of a reference, to matters such as the clarity or validity of a statement or argument. Occasionally Editors' suggestions or criticisms will be considered to be ill-conceived, but authors should realize that if they are misunderstood by Editors they may well be misunderstood by other readers of the Journal. If authors wish to reply to criticisms, their replies will be seriously and sympathetically considered.

Although it is desirable that Editors and referees should be anonymous, it is sometimes a help if direct discussion can take place between an author and an Editor or a referee. This can be arranged at the instance of the author, the Editor or the referee, after consultation with the Chairman.

PREPARATION OF PAPERS*

Full-length papers. Papers submitted for publication in the *Biochemical Journal* should be sent with two abstracts (see below) to the Secretary to the Editorial Board, The Biochemical Journal, 20 Park Crescent, London, W. 1. All communications about reprints should be addressed to the Administrative Secretary, The Biochemical Society, 20 Park Crescent, London, W.1.

Papers submitted should be written concisely. Special attention is directed to the sections below concerning the preparation of the typescript. Strict observance of these requirements will shorten the interval between the receipt of a paper and its publication. Typescripts that are not concise or do not conform to the conventions of the *Biochemical Journal* will be returned to the authors for revision. If a paper that has been returned to an author for revision is not resubmitted within one month, it will, on resubmission, be deemed to be a new paper and the date of receipt altered accordingly. A revised paper containing a significant amount of new material will also be redated.

Submission of a paper to the Editorial Board implies that it reports unpublished work, that it is not under consideration for publication elsewhere, and that if accepted for the *Biochemical Journal* it will not be published elsewhere in the same form, either in English or in any other language, without the consent of the Editorial Board.

Typescripts should bear the name and address of the person to whom the proof of the paper is to be sent.

Papers should be headed by an informative title, by the names of the authors and by the name and address of the Laboratory where the work was performed. Details of financial support may appear in the acknowledgements at the end of the paper. Authors should give a shortened version of the title, not exceeding 45 letters and spaces, suitable for a running title in the published pages of the work.

* For index to pp. 2–9 see under Abbreviations, symbols and conventions (p. 10).

The numbering of parts in a series is no longer permitted. One of the reasons for this decision is that, in the course of time, the main title may become only partly true and thus need modification; also, the practice of numbering papers often results in titles that obscure the true nature of the work rather than reveal it. Titles and subtitles may, however, be used as before.

A paper should be written only when a piece of work is rounded off. Preliminary or abortive experiments should not be described.

It would help the Editors if the author, when submitting a paper, would enclose reprints of relevant preceding papers, especially if they are not published in the *Biochemical Journal*.

Form of papers submitted for publication. Papers should be in double-spaced typing throughout (including References and legends of Tables and Figures) on sheets of uniform size with wide margins. Top copies only should be submitted.

Before preparing a paper authors should consult a current issue of the Journal in order to make themselves familiar with the typographical conventions, use of cross-headings, lay-out of Tables, citation of References etc. The need for editorial revision of badly prepared typescripts or diagrams will delay publication.

Papers on specialized subjects should be presented so that they are intelligible to the ordinary reader of the Journal. Sufficient information must be included to permit repetition of the experimental work.

Generally, papers should be divided clearly into the following sections: (*a*) **synopsis**, not more than 3–4% of the length of the paper; it may be divided into numbered sections; (*b*) **introduction**, containing the reasons for doing the work; (*c*) **Materials and methods**: in chemical papers this section may appear towards the end; (*d*) **Results**: these should be given concisely; the use of both Tables and Figures to illustrate the same results will only rarely be permitted; (*e*) **Discussion**: the presentation of results should be separated from a discussion of their significance; this section should be strictly

limited to discussion and should not recapitulate results; (*f*) acknowledgements; (*g*) References. The arrangement suggested for sections (*c*)–(*e*) is not binding on authors; another arrangement may be more suitable.

The *Biochemical Journal* uses as a standard for spelling the *Concise Oxford Dictionary of Current English* (Oxford: Clarendon Press).

Short Communications. The *Journal* includes, monthly, a limited number of short communications. These generally appear in print about two months after the date of receipt of the typescripts. Typescripts should be submitted *in duplicate*, written in English, and conforming strictly to the form of the *Journal* as far as spelling and abbreviations are concerned. Such communications must not exceed 1200 words of text, but in addition may include either one Table, typed on a separate sheet, or one Figure, drawn according to the rules of the *Journal*. If no Figure or Table is included the text may be expanded to 1400 words. In order to achieve the most rapid publication, articles should arrive at the Editorial Office between the 14th and the 25th day of each month. They should be addressed to the Secretary to the Editorial Board, The Biochemical Journal, 20 Park Crescent, London, W.1, from whom sheets of more detailed instructions for preparing the manuscripts can be obtained. Typescripts that are not to be published will be returned to the authors with the minimum delay. *Authors should submit short papers for this category of publication only when they believe that the rapid communication of their results is of the utmost importance to other investigators.*

Abbreviations and symbols. In general the *Biochemical Journal* follows the Report by the Symbols Committee of the Royal Society on Symbols, Signs and Abbreviations recommended for British Scientific Publications (1951, amended 1959) published in the *Handbook for Chemical Society Authors* (1961), p. 164. Full points are used after abbreviations in lower-case letters for all units. Temperature on the centigrade scale is shown simply as °, not °C. The abbreviations 'hr.' and 'sec.' are used, and mμ and $\mu\mu$ as prefixes to indicate $\times 10^{-9}$ and $\times 10^{-12}$ respectively. Attention is also drawn to *I.U.P.A.C. Information Bulletin*, no. 20 (1963).

New abbreviations should be coined only for unwieldy names, and should not be used at all unless those names occur frequently. All abbreviations for names of compounds except those listed below must be defined together in a footnote.

In the title and synopsis, abbreviations should be avoided. Abbreviations for units (e.g. min., cm., g.) should be used throughout. Chemical symbols as abbreviations for elements, groups or compounds are used only in the Experimental section, and in legends to Tables and Figures, unless their use elsewhere is justified by special circumstances. The abbreviation for the plural of a unit is the same as that for the singular, unless confusion is likely to arise. Thus 'centimetres' is 'cm.', *not* 'cms.'.

C_{20} acid is used to denote an acid containing 20 carbon atoms and C-3 or $C_{(3)}$ to denote the carbon atom numbered 3.

Accepted abbreviations that may be used without definition:

ADP, CDP, GDP, IDP, UDP	5′-Pyrophosphates of adenosine, cytidine, guanosine, inosine, uridine
AMP etc.	Adenosine 5′-phosphate etc.
ATP etc.	Adenosine 5′-triphosphate etc.
CM-cellulose	Carboxymethylcellulose
CoA and acyl-CoA	Coenzyme A and its acyl derivatives
DEAE-cellulose	Diethylaminoethylcellulose
DNA	Deoxyribonucleic acid
DNP-	2,4-Dinitrophenyl-
EDTA	Ethylenediaminetetra-acetate
FAD	Flavine–adenine dinucleotide
FMN	Flavine mononucleotide
GSH, GSSG	Glutathione, reduced and oxidized
NAD	Nicotinamide–adenine dinucleotide*
NADP	Nicotinamide–adenine dinucleotide phosphate*
NMN	Nicotinamide mononucleotide
P_i, PP_i	Orthophosphate, pyrophosphate
RNA	Ribonucleic acid
tris	2-Amino-2-hydroxymethylpropane-1,3-diol

* In support of the recommendations in *Enzyme Nomenclature* (1965) and in *I.U.P.A.C. Information Bulletin*, no. 20 (1963), the Editorial Board will no longer accept 'di-' or 'triphosphopyridine nucleotide' (DPN or TPN).

Oxidized and reduced forms of the dinucleotides should be indicated as, for example, *either* NAD^+, NADH, *or* NAD, $NADH_2$, *not* NAD, NADH. The NAD^+ form is preferred and has the advantage that NAD can be used when the state of oxidation need not be indicated.

Abbreviations for amino acids and sugars. These are for use only in representing polymers or sequences and in Tables and Figures, and need not be defined. In polymers or sequences they should be joined by hyphens if the sequence is known, or by commas if it is not; e.g.:

Gly-Ile-Gly-Phe-(Gly,Tyr,Val,Ser)-Leu-Val-Ala

represents an undecapeptide composed of four amino acids whose sequence has been established,

four for which the sequence is unknown and then three in known sequence. The glycine on the left carries the free amino group and the alanine on the right the free carboxyl group. See *I.U.P.A.C. Information Bulletin*, no. 20 (1963).

Amino acids

Ala	Alanine
Arg	Arginine
Asp(NH_2)	Asparagine
Asp	Aspartic acid
CySH	Cysteine
CyS	Cystine (half)
Glu	Glutamic acid
Glu(NH_2)	Glutamine
Gly	Glycine
His	Histidine
Hyl	Hydroxylysine
Hyp	Hydroxyproline
Ile	Isoleucine
Leu	Leucine
Lys	Lysine
Met	Methionine
Orn	Ornithine
Phe	Phenylalanine
Pro	Proline
Ser	Serine
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine

Sugars

Ara	Arabinose
dRib*	Deoxyribose
Fru	Fructose
Gal	Galactose
Glc	Glucose
Man	Mannose
Rib	Ribose
Xyl	Xylose

* Similarly for other deoxy sugars.

Abbreviations for polynucleotides. Abbreviations should be defined in a footnote at the first mention. If different abbreviations are used, they should be based on the recommendations in *I.U.P.A.C. Information Bulletin*, no. 20 (1963). The preferred system uses capital letters for ribonucleosides:

A	Adenosine	T	Ribosylthymine
C	Cytidine	U	Uridine
G	Guanosine	ψU	5-Ribosyluracil
I	Inosine	X	Xanthosine

The letter p to the left of a nucleoside symbol indicates a 5′-phosphate; to the right it indicates a 3′-phosphate, e.g.:

pApG	5′-*O*-Phosphoryladenylyl-(3′-5′)-guanosine *or* guanylyl-(5′-3′)-adenosine 5′-phosphate
ApGp	Adenylyl-(3′-5′)-guanosine 3′-phosphate
ApG-cyclic-p	Adenylyl-(3′-5′)-guanosine 2′,3′-cyclic phosphate

Other points of attachment of the phosphate groups may be indicated by numerals, e.g.:

A-2′-p-5′-G-2′-p	Adenylyl-(2′-5′)-guanosine 2′-phosphate
ApG-(mixed 2′,3′)-p	A mixture of ApGp and ApG-2′-p

Deoxyribonucleotides are distinguished by the prefix d:

dAp	Deoxyadenosine 3′-phosphate
dTp	Thymidine 3′-phosphate
dApT	Deoxyadenylyl-(3′-5′)-deoxythymidine

Abstracts. For the convenience of authors, two extra copies of the synopsis can be submitted as abstracts. Authors are asked to submit with their typescript two such abstracts suitable for inclusion in *International Abstracts of Biological Sciences* and *Chemical Abstracts*. These abstracts will not appear in the *Biochemical Journal* but will be edited before being passed for publication. An abstract should be typed in double spacing on a separate quarto sheet in the following form: title, name(s) of author(s); *Biochem. J.* (space for year, volume and page reference); address (for reprint applications); synopsis (see p. 2).

Acknowledgements. These must be as short as possible.

Animals. The strain, and if possible the source, of laboratory animals should be stated.

Centrifuging. When conditions for centrifuging are critical, sufficient information should be given to enable the procedure to be repeated. In addition to centrifugal force, ***g***, and the time at maximum speed, the make of centrifuge and rotor used should be given. The centrifugal force quoted should normally be the average ($g_{av.}$), which is based on the maximum speed attained and the radius from the centre of the rotor to the centre of the column of liquid.

Ultracentrifuge data. Sedimentation coefficient (*not* constant), S; sedimentation coefficient corrected to 20° in water, $S_{20,w}$; sedimentation coefficient at zero concentration, S^0, $S^0_{20,w}$; Svedberg unit (10^{-13} sec.), s; partial specific volume, $\bar{v}$; diffusion coefficient, D, D^0, $D_{20,w}$ etc. as for sedimentation coefficient. The temperature at which the sedimentation and diffusion measurements are made should be stated.

Chromatograms. Photographs or drawings of paper chromatograms, particularly one-dimensional, are not generally published unless they show some feature that cannot be described in the text. In paper and thin-layer chromatography, the rate of movement of a substance relative to that of the solvent front is expressed as R_F, or relative to a reference compound as R_X, where X is an appropriate abbreviation for the reference compound. Solvents should be described in the form butan-1-ol–acetic acid–water (4:4:1, by vol.) or butan-1-ol–acetic acid (4:1, v/v).

Dialysis. The terms 'diffusate' and 'non-diffusible material' (or 'dialysis residue') should be used. 'Dialysate' should not be used.

Enzymes. The recommendations of *Enzyme Nomenclature* (1965; Elsevier Publishing Co.) will be followed as far as possible.

Footnotes. These should be avoided as far as possible (except in the definition of abbreviations). Where they must be used, as in Tables, reference is made by the symbols * † ‡ § ‖ ¶, in that order.

Illustrations. These constitute an expensive item in publication and may increase the time taken in printing. Their number should be kept to a minimum. Illustrations requiring reproduction as half-tone plates should be avoided whenever possible; they may be accepted if their publication is important to the understanding of the text. Diagrams that do not conform to the directions given below [see *Notes on Preparation of Illustrations, Biochem. J.* (1956) **62**] will have to be redrawn. Illustrations should be on separate sheets and packed flat; they should bear the author's name, the title of the paper and the Figure number on the back. Their approximate position should be indicated in the margin of the typescript.

Histograms. Simple histograms recording only a few values should not be used. The information can be given more concisely and compactly in a Table.

Legends. Legends and captions should be typed on a sheet of paper separate from the illustrations and numbered correspondingly. They should be written so that the general meaning of each illustration can be understood without reference to the text; reference to the text for the description of general experimental details is permissible, but conditions specific to the particular experiment should be stated.

Lettering. All lettering on Figures must be left to the printer; guide lettering should be done in soft pencil. Individual curves should be distinguished by distinctive symbols (see below), single-letter labels or distinctive line forms. Explanatory labels within a Figure should be avoided.

Materials. Diagrams should be in indian ink and should preferably be drawn on white card, faintly blue- or green-lined graph paper or tracing cloth or paper. Mounting on heavy cardboard is undesirable.

Size. Illustrations should be approximately twice the size of the finished block (usually single-column width). A margin of at least 3 cm. is essential. The card, cloth or paper on which the drawing is made should not exceed foolscap size (33 cm. × 21 cm.). If drawings larger than 33 cm. × 21 cm. are unavoidable they must be accompanied by smaller photographic copies for the use of Editors. In a drawing of apparatus and in a photomicrograph the scale must be indicated.

Symbols for experimental points. The preferred symbols are ○, △, □, ●, ▲, ■. The same symbols must be not be used on two curves where the points might be confused. The symbols × or + should be avoided. For scatter diagrams filled-in symbols are preferred.

Technique. All curves, lines and symbols should be drawn with instruments. Curves should not be drawn beyond experimental points. Scale marks must be within the axes of a graph. Axes should not extend appreciably beyond the curves. It is sometimes unnecessary for an axis scale to start at 0; only the part of the scale relevant to the curves should be given.

Shading and hatching should be left to the printer; areas to be shaded or hatched should be indicated lightly in blue pencil.

Line thickness must be uniform and appropriate to the size of the drawings. For an original of 10 cm. × 10 cm. this should be 0·5–0·6 mm. and for one of 15 cm. × 15 cm. it should be 0·7–0·9 mm.

Isotope experiments. The information given should include: (*a*) sufficient details of the method of assay to allow an estimate of the efficiency of detection (preferably an assay of a standard under the same conditions); (*b*) details of corrections made to the observed count rate; (*c*) form of the sample, e.g. infinitely thick, infinitely thin; (*d*) standard error of the results or a statement of the total counts above background collected; (*e*) in general the specific activity of the starting and final materials should be given, preferably in terms of curies per unit weight or, for stable isotopes,

as atoms per cent excess. For some purposes the count rate under defined conditions such as at infinite thickness is satisfactory, but authors should consider any limitations that such statements may impose on the deductions from their work.

In assessing the specific activities of starting materials, dilution with unlabelled materials in the incubation mixture should be allowed for. This is not always possible but, unless the dilution is known, the radioactivity measurements do not indicate the amount of material transferred.

Metabolic quotients and enzyme units. It has been customary to use the symbol Q_x to indicate μl. of gas X/mg. dry wt. of biological material/hr.; metabolic quotients in any other units have been represented by q_x. As far as possible this notation will be discontinued and units should be stated in full each time they are used. Units of enzyme activity should be given as μmoles of substrate transformed/min., or if necessary as μmoles of the measured product/min. Similarly metabolic quotients should, if possible, be given as μmoles/min. for a defined arbitrary quantity of material, e.g. mg. dry wt., mg. of protein, g. wet wt. etc. The temperature and other conditions should always be stated. The term 'optimum conditions' is not acceptable.

Michaelis constant. This is defined as $K_m = [S]$ when $v = V/2$, where v is the velocity of appearance of product or disappearance of substrate at a given substrate concentration [S] and V is the velocity when the enzyme is saturated with substrate. When reactions with two substrates A and B are being considered $K_m^A = [A]$ when $v = V/2$ and [B] is at a saturating concentration, and $K_m^B = [B]$ when $v = V/2$ and [A] is at a saturating concentration. K_s, K_s^A and K_s^B are the equilibrium dissociation constants.

Micro-organisms. In the title, in the synopsis and at the first mention in the text, micro-organisms should be given their full binominal Latin name and underlined. If the organism has been obtained from a recognized collection of micro-organisms, a collection number must be given; alternatively, a strain number or name should be quoted; this should not be underlined. Names of ranks higher than genus (e.g. Eubacteriales, Lactobacilleae), generic names used adjectivally (e.g. 'staphylococcal') and names of micro-organisms used colloquially (e.g. as in 'most lactobacilli behave thus') should not be underlined. The first (i.e. generic) name should be spelt with a capital letter. Elsewhere in the text, single-letter abbreviations may be given for the generic name; if two genera with the same initial letter are studied, abbreviations such as *Strep.* and *Staph.* may be used. If the author selects for stated reasons a name that does not conform to that chosen in the most recent edition of one of the reference books quoted below, the name given in the reference book should be added in parenthesis after the first mention of the organism in the synopsis, and also in the text. Characteristics of the organism that are known to differ from those quoted in the reference book should also be given, since they are essential for subsequent interpretation of the work.

Authors are urged to offer organisms that have uncommon characteristics or are valuable for specific purposes to collections of micro-organisms so that they may be readily available to other workers. The addresses of the British collections of micro-organisms are obtainable from the Editorial Office.

Reference books. Bergey's *Manual of Determinative Bacteriology*. London: Baillière, Tindall and Cox. *A Dictionary of the Fungi* by Ainsworth, G. C. & Bisby, G. R. Kew: Imperial Mycological Institute. *The Yeasts: a Taxonomic Study* by Lodder, J. & Kreger van Rij, N. J. W. Amsterdam: North Holland Publishing Co.

Moles. The word 'mole' is used with the meaning of 'gram-molecule'. It must not be abbreviated to 'mol.' or 'M'. The submultiples will be printed as 'm-mole', 'μmole' and 'mμmole'.

Powers in Tables and Figures. Care is needed where powers are used in Table headings and in Figures in order to avoid numbers with an inconvenient number of digits. The quantity expressed is to be preceded by the power of 10 by which its value has been multiplied. The units in which the quantity is expressed may not be multiplied by a power of 10; the unit may be changed by the use either of prefixes, such as m or μ, or of another accepted scale such as Å. For example: (i) An entry '2' under heading 10^3k means that the value of k is 0·002; an entry '2' under heading $10^{-3}k$ means that the value of k is 2000. (ii) A concentration 0·00015 M may be expressed as 0·15 under heading 'concn. (mM)' *or* as 150 under heading 'concn. (μM)' *or* as 15 under heading '$10^5 \times$ concn. (M)', but *not* as 15 under heading 'concn. ($M \times 10^{-5}$)'. (iii) Complex quantities are similarly treated; a value for $1/[S]$ of 200 M^{-1} would appear as '2' under the heading $10^{-2}/[S]$ (M^{-1}).

References. These should be given in the text thus: Barnett & Robinson (1942), (Culbertson & Thomas, 1933). Where part of the volume is published in one year and part in the next year, the year or years should be given as on the title page

of the volume. When a paper to be cited has more than two authors, the names of all the authors should be given when reference is first made (except for more than five authors), e.g. (Osborne, Mendel & Ferry, 1919); subsequent citations should appear thus: (Osborne *et al.* 1919). Where more than one paper by the same authors has appeared in one year the reference should be given as follows: Osborne & Mendel (1914*a*); Osborne & Mendel (1914*b*); or Osborne & Mendel (1914*a*,*b*); (Osborne & Mendel, 1914*a*, 1916; Barnett & Robinson, 1942).

At the end of the paper references should be given in alphabetical order in *double-spaced type throughout*, and should include the authors' initials, but not the title of the paper, thus:

Barnett, J. W. & Robinson, F. A. (1942). *Biochem. J.* **36**, 364.

Titles of journals should be abbreviated in accordance with the system used in the *World List of Scientific Periodicals* (1952, 3rd ed. London: Butterworths Scientific Publications). References to books and monographs should include the town of publication and the name of the publisher, as well as the date of publication and the number of the edition to which reference is made. Examples are:

Dixon, M. & Webb, E. C. (1964). *Enzymes*, 2nd ed., p. 565. London: Longmans, Green and Co. Ltd.

Holman, R. T. & Bergström, S. (1951). In *The Enzymes*, vol. 2, part 1, p. 559. Ed. by Sumner, J. B. & Myrbäck, K. New York: Academic Press Inc.

Solutions. Solutions of common acids and bases should be described in terms of normality (N), and salts preferably in terms of molarity (M), e.g. N-HCl, 0·1 M-NaH_2PO_4, 0·1 N-acetic acid. Fractional concentrations should be expressed in the decimal system, e.g. 0·25 N-HCl (*not* N/4 HCl). The term % must be defined as w/w, w/v or v/v, e.g. 5% (w/v) means 5 g./100 ml. For aqueous solutions of concentration less than 1%, w/v need not be inserted if it is clear that the concentration is stated in terms of weight of solute. For solutions of salts expressed as % it must be made clear whether anhydrous or hydrated compounds are used.

Buffers. Cationic and anionic composition and pH should be given. An example of a suitable description is '0·1 M-KH_2PO_4 adjusted to pH 7·4 with 2 N-NaOH'. Alternatively a reference should be given. It should always be made clear whether concentrations of ingredients in a reaction mixture are final concentrations or the concentrations of solutions added.

Krebs–Ringer solution should be described by reference or the composition should be given.

The symbol for ionic strength is I.

Spectrophotometric data. For spectrophotometric terms, symbols and curves see *Handbook for Chemical Society Authors* (1961), p. 176.

Extinction ($\log I_0/I$) should be used, *not* absorbancy *and not* optical density. Abbreviations used are: E, extinction; $E^{1\%}_{1\,cm.}$, specific extinction coefficient; ϵ, molar extinction coefficient (the extinction of a molar solution in a 1 cm. light-path).

Curves showing absorption in the ultraviolet or visible region should always have a wavelength scale, whether or not a wave-number scale is included.

Statistical treatment of results. It is often unnecessary to publish the individual results of a number of similar experiments. When the object is to determine the value of a quantity or the statistical characteristics of a population, sufficient information is usually conveyed by the following: (i) the number of individual experiments; (ii) the mean value; (iii) the standard deviation (S.D.), the coefficient of variation, or the standard error of the estimate of mean value (S.E.M.) as may be appropriate. *It should be made clear whether the standard deviation or the standard error is used.* A convenient form for inclusion in a Table is, for example $263 \pm 2{\cdot}5$ (10), where the number in parenthesis represents the number of results.

Where a significant difference is claimed between the means (or other statistics) of two groups of results, the test of significance used should be stated.

In representing statistical quantities by symbols, the convention of using Greek letters (Σ, σ, μ, etc.) for the hypothetical characteristics of the population, and Roman letters (S, s, m, etc.) for actual estimates of their values based on limited samples, should be observed.

Tables. Tables should have legends which make their general meaning comprehensible without reference to the text. Footnotes should be kept to a minimum. Conditions specific to the particular experiment should be stated. Reference to the text for general experimental methods is permissible provided that there is no ambiguity. The units in which the results are expressed, e.g. g./100 ml., should be given at the top of each column, and not repeated on each line of the Table.

Tables should be typed on separate sheets and their approximate position in the text indicated. Words or numerals should be repeated on successive lines: 'ditto' or ',,' is not to be used.

Trade names. The names of the manufacturers or suppliers of special apparatus or materials should be given, and also their addresses where these are outside Great Britain or not well known. Wherever possible, the chemical nature of proprietary material should be specified at the first mention.

CHEMICAL NOMENCLATURE

The system of The Chemical Society is usually followed. The fundamental principles of organic and inorganic chemical nomenclature are given by the I.U.P.A.C. 1957 Rules (Butterworths Scientific Publications, London, 1958); these are given in *Handbook for Chemical Society Authors* (1961), pp. 46–163. For detailed proposals for the nomenclature of amino acids, see *Handbook for Chemical Society Authors*, p. 186. Definitive names for oligosaccharides are often too cumbersome for repeated mention in the text of a paper. The Editorial Board have sanctioned the use of shortened names for oligosaccharides which, within the conventions of the system employed, are unambiguous. For examples see *Biochem. J.* (1956), **63**, 200; **64**, 340; **64**, 351. These abbreviated names are not to be considered as superseding the internationally agreed names. At its first mention the shortened name of the oligosaccharide should be given in parentheses after the definitive names based on the Rules of Carbohydrate Nomenclature (*Handbook for Chemical Society Authors*, p. 140, and *Editorial Report on Nomenclature*, 1962, published by The Chemical Society).

Analyses and physical properties. Standard forms for reporting these are:

The *new compound* (name in italics) had m.p. 175° (decomp.), $[\alpha]_D^{22} + 17 \pm 2°$ (*c* 1·6 in water), light-absorption max. in ethanol 226 and 265 mμ (ε 2200 and 2500 respectively) (Found: C, 40·8; H, 6·9; N, 11·5; OMe, 26·0; $C_8H_{16}N_2O_6$ requires C, 40·7; H, 6·8; N, 11·9; OMe, 26·3%).

The known compound (name in roman type) had m.p. 178–179°, unchanged by admixture with an authentic sample kindly supplied by Dr Z (Found: C, 48·6; H, 6·1; OMe, 50·1. Calc. for $C_{10}H_{16}O_7$: C, 48·4; H, 6·4; OMe, 50·0%). *Or*: The known compound had m.p. 178–179°. The mixed m.p. with an authentic sample (m.p. 179–181°) prepared according to X & Y (1932) was 178–180° (Found: C, 49·4; H, 3·8; N, 3·9; loss at 100°, 5·1. Calc. for $C_{28}H_{22}I_2N_2,2H_2O$: C, 49·7; H, 3·9; N, 4·2; H_2O, 5·3%). (If water of crystallization is claimed, evidence should be given, e.g. as loss at 100° as above, or the reason why it cannot be given should be explained.)

Distillation of the product gave a middle fraction (0·3 g.), b.p. 120°/15 mm., n_D^{16} 1·4767.

Elementary analyses. Percentages should generally be given to one place of decimals only. Elements are to be listed in the order C, H and then the remainder in alphabetical order of symbols.

Melting points. It is desirable to state whether these are corrected or uncorrected for the emergent stem of the thermometer. This can be done at the beginning of the Experimental section.

Specific optical rotations. An estimate of the error should be given.

Formulae. These should, if possible, be written in one line, as this saves space and expense in printing. Dots are used to represent the links in the main chain; side chains are in parentheses, and condensed main chains are in square brackets, e.g.

$$CH_2{:}CH{\cdot}CH(OH){\cdot}CH_3$$
$$H_2N{\cdot}[CH_2]_3{\cdot}CH(NH_2){\cdot}CO_2H$$

Formulae with rings or branched chains should be clearly written on a separate sheet so that they can be copied by the draughtsman. Hetero atoms should be shown in the ring, and aromatic rings must show double bonds.

R, R′, R″ (or R^1, R^2, R^3, R^4 if more than three) should be used to denote variable substituents in formulae.

Hyphens. All chemical names are run together except for those of acids, acetals, esters, ethers, glycosides, ketones and salts, which are printed as separate words; hyphens are used to separate numbers, Greek letters or some configurational and italic prefixes from words, e.g. *m*-dinitrobenzene, ββ-dimethyl-D-cysteine, 2-*p*-isopropylphenylheptane, ethyl methyl ketone (butan-2-one).

Ions. These should be represented thus: Na^+, Zn^{2+}, Cl^-, PO_4^{3-}.

Isotopically labelled compounds. The following conventions were adopted by the Editorial Board of The Biochemical Society and the Editors of The Chemical Society (see *Handbook for Chemical Society Authors*, p. 186).

The symbol for the isotope introduced is placed in *square* brackets directly attached to the front of the name, as in [^{14}C]urea. When more than one position in a substance is labelled by means of the same isotope the number of labelled atoms is added as a right-hand subscript, as in [$^{14}C_2$]glycollic acid. The symbol U indicates 'uniform' and 'G' general labelling, e.g. [U-^{14}C]glucose, [G-^{14}C]glucose.

The isotopic prefix precedes that part of the name to which it refers, as in 2-acetamido-7[^{131}I]-iodofluorene, α-naphth[^{3}H]oic acid ($C_{10}H_7{\cdot}CO_2{}^3H$), sodium [^{14}C]formate, 1-amino[^{14}C]methylcyclopentanol ($H_2N{\cdot}{}^{14}CH_2{\cdot}C_5H_8{\cdot}OH$).

When isotopes of more than one element are introduced, their symbols are arranged in alphabetical order, including ^{2}H and ^{3}H for deuterium and tritium respectively.

When not sufficiently distinguished by the foregoing means, the positions of isotopic labelling are indicated by Arabic numerals, Greek letters, or prefixes (as appropriate), placed within the square brackets and before the symbol of the element concerned, to which they are attached by a hyphen; examples are [1-$^{2}H_{1}$]ethanol ($CH_3 \cdot CH^2H \cdot OH$), [1-^{14}C]aniline, [$\alpha$-^{14}C]leucine, [*carboxy*-^{14}C]leucine, [*Me*-^{14}C]isoleucine, [6,7-$^{14}C_2$]xanthopterin, [$\alpha\beta$-$^{14}C_2$]maleic anhydride, [$\beta\gamma$-$^{13}C_2$,^{35}S]methionine, [1-^{14}C,2-^{13}C]acetaldehyde, [β-^{14}C,$\alpha\beta$-$^{2}H_2$,^{15}N]serine.

Italics. These are used for certain prefixes that will not be counted as part of the name for indexing, e.g. *n-*, *sec.-* (*not* s-), *tert.-* (*not* t-), *cis-*, *trans-*, *o-*, *m-*, *p-*, *dextro*, *laevo*, *meso*. Italics are also used for *O-*, *N-* etc. to indicate an element carrying a substituent, e.g. N^4-acetylsulphanilamide. Italics should *not* be used for allo, bis, cyclo, epi, iso, neo, nor, tris.

Optically active isomers. Small capital letters, D-, L- and DL-, are used for optically active compounds and their racemates when the compounds can be correlated sterically with glyceraldehyde or serine. In all other cases (+)-, (−)-, (±)-, or *dextro*, *laevo* and 'optically inactive', are used.

Prefixes denoting substituents. An alphabetical order will be followed (cf. *Handbook for Chemical Society Authors*, pp. 52, 184). Syllables indicating multiple substituents, e.g. di-, tri-, do not count in deciding the order.

ABBREVIATIONS, SYMBOLS AND CONVENTIONS

This list includes accepted symbols and abbreviations apart from those already listed on pp. 3 and 4 and also serves as an index; definitions are included that may be of help to authors.

acceleration due to gravity	*g*
adenosine triphosphatase	ATPase *allowed if defined*
adenosine triphosphate	the three phosphorus atoms are distinguished as α, β and γ, thus: adenosine–P^{α}–O–P^{β}–O–P^{γ}
aliquot	*not used*; *substitute* sample, portion *or* fraction
alternating current	a.c.
amino acids, abbreviations for	p. 4
ampere	A
analytical standard of purity	A.R.
aneurin	*not used*; *use* thiamine, *alternative permitted* vitamin B_1
Ångström unit	Å
approximately	approx. (*or use* about, *not c. or ca.*)
aqueous	aq.
ascorbic acid	*alternative permitted* vitamin C
atmosphere	atm.
atomic weight	at.wt.
axial ratio (of spheroid)	preferably expressed as *a*/*b* (semi-axis of rotation = *a*, semi-equatorial axis = *b*)
Basal Metabolic Rate	BMR *allowed if defined*
boiling point	b.p.
British Pharmacopoeia	B.P. (with date)
buffers	p. 7
calciferol	*use* ergocalciferol, *alternative permitted* vitamin D_2
calculated	calc.
calorie (small), gram-calorie	cal.
Calorie (large), kilogram-calorie	*not used*; *use* kcal.
capric acid	*use* decanoic acid
caproic acid	*use* hexanoic acid
caproyl	*use* hexanoyl
capryl, caprinoyl	*use* decanoyl
caprylic acid	*use* octanoic acid
caprylyl, capryloyl	*use* octanoyl
carbobenzyloxy	*use* benzyloxycarbonyl
centimetre	cm.
centimetre gram second	c.g.s.
cerebrospinal fluid	c.s.f. *allowed if defined*
cocarboxylase	*use* thiamine pyrophosphate
coefficient of variation	standard deviation/mean value (*see* p. 7)
compare	cf.
concentrated	conc.
concentration	concn.
concentration (symbol, e.g. in specific rotation)	*c*
constant, equilibrium	*K*
constant, velocity	*k*
corrected (e.g. m.p., for emergent stem)	corr.
counts/min.	*not* c.p.m.
crystalline, crystallized	cryst.
cubic	cu. *or as* e.g. $mm.^3$

curie	c ($3{\cdot}77 \times 10^{10}$ disintegrations/sec.)
cycles per second	cyc./sec.
decomposition (m.p.)	decomp.
degrees (temperature)	* °C, °K, °F
deoxy (prefix)	*not* desoxy
deoxyribonucleosides, abbreviations for	p. 4
dialysable	*not permitted*; *use* diffusible (*see* p. 5)
dialysate	*not used*; *for* diffusible material *use* diffusate (*see* p. 5)
diffusion coefficient	D, $D_{20,w}$, D^0 etc. (*as for* sedimentation coefficient) (*see* p. 5)
dilute	dil.
direct current	d.c.
disintegrations/min.	*not* d.p.m.
dissociation constant, minus log of	pK
disulphide group	*alternative permitted* S·S group
dithionite (sodium)	$Na_2S_2O_4$, *not* hydrosulphite, hyposulphite
dry ice	*use* solid CO_2
electrode potential, standard	E_0
electrode potential, standard at constant pH	E'_0
electromotive force	e.m.f.
electron volt	ev
enzyme units	p. 6
equation	eqn.
equivalent (weight)	equiv.
ethanol, ethanolic	*not* ethyl alcohol, *not* alcoholic
Experiment (with reference numeral)	Expt.; *pl.* Expts.
farad	F
Faraday	F
Figure (with reference numeral)	Fig.; *pl.* Figs.
Figures, preparation of	p. 5
foot	ft.
formulae	p. 8
free energy (Gibbs)	G
frictional coefficient (molar)	f
frictional coefficient (molar) for sphere of same volume	f_0
gallon (imperial)	gal. (= 4·55 l.)
gas constant per mole	$\boldsymbol{R}$
α-glycerophosphoric acid	*use* L-3-glycerophosphoric acid when the configuration is to be specified
glycerophosphorylcholine	GPC *allowed if defined*
glycerophosphorylethanolamine	GPE *allowed if defined*
glycerophosphorylserine	GPS *allowed if defined*
glyoxaline	*not used*; *use* imidazole
gram	g.
gram-atom	g.atom
gram-calorie	cal.
gram-equivalent	g.equiv.
gram-molecule	mole
gravity, centrifugal	$\boldsymbol{g}$ (*see* p. 4)
haem, protohaem	prosthetic group of haemoglobin
haematin, protohaematin	oxidized haem
haemochromogen	haem + base *or* haem + denatured protein
half-wave potential (polarography)	$E_{\frac{1}{2}}$
hour	hr.
hydrogen-ion concentration, minus log of	pH, *pl.* pH values
hydroquinone	*use* quinol

* Centigrade scale assumed in absence of indication; c to be inserted only when more than one scale is used.

hydrosulphite, hyposulphite	*not used*; *see* dithionite
imidazole	glyoxaline *not used* (*not* iminazole)
inch	in.
infrared	i.r.
inhibitor constant	K_i (dissociation constant of inhibitor–enzyme complex)
insoluble	insol.
international unit	i.u.
ionic strength	I
isoenzyme	*not* isozyme
isotonic	specify composition of solution, e.g. *use* 0·9% NaCl solution
isotopically labelled compounds	pp. 8 and 9
joule	J
junior	jun.
kephalin	amino phospholipids *preferred*
keto acid	keto used only generically, *otherwise* oxo
keto sugars	*use* pentulose, hexulose etc., *not* ketopentose, ketohexose etc.
Krebs–Ringer solution	reference to be given
light petroleum	*not* petroleum ether: boiling range to be stated
lipid	*not* lipide, lipin, lipoid
litre	l.
logarithm, in text	log
logarithm (base 10), in formulae	log
logarithm (base e), in formulae	ln
maximum	max.
median effective dose	ED_{50}
median lethal dose	LD_{50}
melting point	m.p.
metabolic quotients	*see* p. 6
methanol, methanolic	*not* methyl alcohol
metre	m.
Michaelis constant	K_m (*see* p. 6)
microgram	μg. (*not* γ)
microgram-atom	μg.atom (*not* μatom)
micromicro- (prefix, $10^{-12}\times$)	$\mu\mu$ (*preferred to* p)
micromole	μmole *not* μM
micron (10^{-6} metre)	μ
milliequivalent	m-equiv.
millilitre	ml.
millimicro (prefix)	mμ (*preferred to* n)
millimicron (10^{-9} metre)	mμ
*millimolar (concentration)	mM
millimole	m-mole *not* mM
minimum, minute	min.
*molar (concentration)	M
molecule, molecular	mol.
molecular weight	mol.wt.
*normal (concentration)	N
normal temperature and pressure	*not used*; *use* standard temperature and pressure
nuclear magnetic resonance	n.m.r.
number (in enumerations)	no.
observed	obs.
ohm	Ω
optical rotation	specific optical rotation (with concn. 1g./ml., light-path 10 cm.), e.g. $[\alpha]_D^{20}$, $[\alpha]_{5461}^{25}$ molecular optical rotation ($=[\alpha]_\lambda^t \times$ mol.wt.), e.g. $[M]_D^{20}$, $[M]_{5461}^{25}$. If a different value, e.g. $[\alpha]_\lambda^t \times$ mol.wt./100, is used, this should be stated

* Separated by a hyphen (and no full stop) from a chemical formula or name following it, e.g. M-NaCl; N-NaOH; N-sulphuric acid.

optically active isomers	p. 9
osmolar	the concentration producing an osmotic pressure equal to that of a molar solution of a perfect solute
ox	domestic bovine species without sexual distinction; *not* beef
page, pages	p., pp.
partial specific volume	$\bar{v}$
parts per million	p.p.m.
per (e.g. grams per litre)	/ (e.g. g./l.)
per cent	%
petroleum ether	*not used* (*see* light petroleum)
phenyl hydrogen sulphate	*not* phenol sulphate *or* phenyl sulphuric acid
phosphatide	phospholipid *preferred*
phosphoglyceryl	*not allowed*; *see* α-glycerophosphoric acid
potassium hydroxide	*not* caustic potash
potential difference	p.d.
pound (weight)	lb.
precipitate	ppt.
preparation	prep.
probability of an event's being due to chance alone	P
pyridoxine, pyridoxal	vitamin B_6 *permitted*
quinol	*not* hydroquinone
reciprocal centimetre	$cm.^{-1}$
recrystallized	recryst.
refractive index	n; at stated temperature and wavelength represent as e.g. n_D^{20}
relative band speed (partition chromatography)	R, R_F, R_X (*see* p. 5); *pl.* R values etc.
respiratory quotient	R.Q. *allowed if defined*
revolutions	rev.
rev./min.	*not* r.p.m.; *use* **g** where possible (*see* p. 4)
riboflavine	vitamin B_2 *permitted*
ribonucleosides, abbreviations for	p. 4
röntgen unit	r.
second (time)	sec.
sedimentation coefficient	S; *not* sedimentation constant (*see* p. 5)
sedimentation coefficient corrected to 20° in water	$S_{20,w}$; S_{20} may be used, if it is unambiguous (*see* p. 5)
sedimentation coefficient, at zero concentration	S^0, $S^0_{20,w}$ etc. (*see* p. 5)
sodium hydroxide	*not* caustic soda
soluble	sol.
solution	soln.
solutions, concentrations of	p. 7
solvent systems	e.g. butanol–acetic acid–water (4:1:1, by vol.); butanol–acetic acid (4:1, v/v)
species (sing. and pl.)	sp., spp.
specific gravity	sp.gr.
square	sq. *or as* e.g. $cm.^2$
standard deviation	S.D. } *see* p. 7
standard error of estimate of mean value	S.E.M. } *see* p. 7
standard temperature and pressure	s.t.p.
standard wire gauge	S.W.G.
substituents (variable, in organic compounds)	R, R′, R″, or R^1, R^2, R^3, R^4 (if more than three) (*see* p. 8)
substrate constant	K_s (dissociation constant of substrate–enzyme complex)

sulphydryl	thiol *or* SH *preferred*
sum	Σ *or* S (*see* p. 7)
Svedberg unit ($10^{13}\times$ value in c.g.s. units)	S (*see* p. 5)
temperature	(abbrev.) temp.; (symbol) t (empirical), T (absolute)
thiamine	*alternative permitted* vitamin B_1
time (symbol)	t
tocopherol	*alternative permitted* vitamin E
trichloroacetic acid	TCA *not used*
turnover number	of an enzyme; number of reaction cycles undergone by each active centre/min.
ultracentrifuge data	p. 5
ultraviolet	u.v.
uncorrected (m.p., for emergent stem)	uncorr.
variety (e.g. botanical)	var.
velocity (symbol)	v
veronal	*used* only for buffer mixtures; *otherwise use* 5,5′-diethylbarbituric acid
viscosity, relative	$\eta_{rel.}$ $\left(\frac{\text{viscosity of solution}}{\text{viscosity of solvent}}\right)$
viscosity, specific	$\eta_{sp.}$ (i.e. $\eta_{rel.}-1$)
viscosity, reduced	$\eta_{sp.}/c$ (units: ml./g.)
viscosity, intrinsic	$[\eta]$, i.e. $\lim_{c\to 0}\eta_{sp.}/c$
volt	v
volume (abbrev. after number)	vol.
v/v	*used* only for two components; by vol. *used* for three or more components
watt	w
wavelength	λ
wavelength of D line of sodium (other wavelengths in Å)	D (as subscript)
wave number (unit)	$cm.^{-1}$
weight	wt.

3-*O*-Methyl-D-mannose from *Streptomyces griseus*

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(*Received 2 June* 1965)

1. *Streptomyces griseus* was grown in a medium containing L-[*Me*-^{14}C]methionine, and the labelled products from an ethanolic extract of the cells were examined. 2. Acid hydrolysis of one of the products gave a compound identified as 3-*O*-[*Me*-^{14}C]-methylmannose by a series of degradative reactions. 3. Reduction of the radioactive compound gave 3-*O*-methyl-D-mannitol, indistinguishable from a synthetic sample.

It has been shown (Candy, Blumsom & Baddiley, 1964) that the methyl group of methionine is incorporated efficiently into the *N*-methyl-L-glucosamine moiety of streptomycin by *Streptomyces griseus*. During unsuccessful attempts to identify intermediates in the biosynthesis of streptomycin, *S. griseus* was grown in the presence of [*Me*-^{14}C]-methionine and the labelled products were investigated. No such intermediates were identified, but during the investigation a highly radioactive fraction was obtained that gave on hydrolysis 3-*O*-methyl-D-mannose.

MATERIALS AND METHODS

Growth of organism. Streptomyces griseus (Glaxo strain L118) was grown on slopes as described by Candy *et al.* (1964). The resulting spores were used to inoculate 100ml. of a medium containing Oxoid Nutrient broth no. 2 (2·5%) and glucose (1%), and the culture was grown for 24hr. on a shaker (140 oscillations/min.). Portions (50ml.) of this culture were used to inoculate each of two flasks (Glaxo pattern, 2·5l. capacity), each containing 200ml. of Carvajal's (1957) medium, 0·2g. of soya-bean meal and 9·6μmoles (0·15mc) of [*Me*-^{14}C]methionine (The Radiochemical Centre, Amersham, Bucks). The cultures were grown for 48hr. on the shaker.

Preparation of extract. The culture was centrifuged at 10000*g* for 20min. at 5° and the sedimented fraction was resuspended in 200ml. of 0·6% NaCl solution and again centrifuged. The sedimented fraction was made into a slurry with water and adjusted to pH6 with 0·2N-HCl. Boiling ethanol (2vol.) was added, the mixture was rapidly brought to the boil and after 1min. it was cooled and filtered.

Fractionation of extract. The extract was evaporated under reduced pressure to 20ml. and passed through a column of Dowex 1 (Cl^- form) resin (9ml.), then through a column of Amberlite CG-50 (Na^+ form) resin (5ml.) and finally through a column of Dowex 50 (H^+ form) resin (11ml.). The solution was neutralized by stirring with Amberlite IR-45 (OH^- form) resin and was evaporated under reduced pressure to a small volume. This was called the 'neutral fraction'.

Hydrolysis of 'neutral fraction'. A sample of the 'neutral fraction' was hydrolysed in N-H_2SO_4 for 16hr. at 100° in a sealed tube. The hydrolysate was diluted, neutralized with $BaCO_3$ and filtered. A paper chromatogram developed in solvent 1 showed that almost all the radioactivity was present in a spot with R_{Glc} 1·8. The radioactive compound was partially purified in the following manner.

The hydrolysate was mixed with a small amount of cellulose powder and applied to a column (1cm. × 37cm.) of Whatman cellulose powder, previously washed with propan-1-ol–ethyl acetate (7:1, v/v). Material was then eluted by successive application of propan-1-ol–ethyl acetate–water (7:1:1, by vol.) (50ml.), propan-1-ol–ethyl acetate–water (7:1:1·5, by vol.) (40ml.) and finally propan-1-ol–ethyl acetate–water (7:1:2, by vol.) (40ml.). Almost all the radioactivity corresponded to a single peak eluted at 60ml. The fractions containing the radioactivity were combined and concentrated under reduced pressure to a small volume.

3-O-*Methyl*-D-*mannose from medium.* The supernatant obtained by centrifugation of the original culture was deionized in the same way as described for the cell extract. On hydrolysis (N-HCl for 16hr. at 100°) this neutral fraction gave a radioactive compound that corresponded chromatographically and electrophoretically to the product of hydrolysis of the 'neutral fraction'.

Paper chromatography. The following solvent systems were used: solvent 1, propan-1-ol–ethyl acetate–water (7:1:2, by vol.); solvent 2, butan-1-ol–ethanol–water (5:1:4, by vol.; top layer); solvent 3, butan-1-ol–ethanol–water–aq. NH_3 (sp.gr. 0·88) (40:10:49:1, by vol.; top layer). Whatman no. 1 paper was employed. Sugars were detected either by the method of Trevelyan, Procter & Harrison (1950) or by the periodate–Schiff reaction (Baddiley, Buchanan, Handschumacher & Prescott, 1956).

Determination of radioactivity. Radioactivity on paper chromatograms was detected either with a strip scanner (Atomic Accessories Inc.) or by radioautography with Kodirex X-ray film. Measurements were made with a

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Packard Tri-Carb scintillation counter no. 3314, as described by Candy & Baddiley (1965).

Preparation of 3-O-*methyl*-D-*mannitol*. Methyl 4,6-*O*-benzylidene-α-D-mannoside was prepared from D-mannose as described by Buchanan & Schwarz (1962). The product (12g., m.p. 144°) was partially methylated by adding a mixture of methyl iodide (20ml.) and methanol (5ml.) and refluxing. Silver oxide (12g.) was added in several portions during 3hr. and refluxing was continued for a further 3hr. After being cooled, the reaction mixture was filtered and the residue was washed with chloroform and then methanol. The combined filtrate and washings were evaporated under reduced pressure, and the residue was hydrolysed in N-HCl (50ml.) for 30min. at 100°. After evaporation under reduced pressure the product was refluxed for 3hr. in methanol containing 0·5% of HCl. The mixture was neutralized with Amberlite IR-45 (OH^- form) resin and filtered, and solvent was evaporated. The residue was dissolved in 0·05M-sodium periodate solution (1l.) and the solution was kept for 24hr. at room temperature in the dark. Isomeric 2-*O*-methylmannoside was oxidized under these conditions. Spectrophotometric examination of the reaction mixture at 223mμ (Dixon & Lipkin, 1954) showed that about 80% of the periodate remained. The reaction mixture was deionized by passage through an excess of Amberlite IR-120 (H^+ form) resin and then Amberlite IR-45 (OH^- form) resin. The solution was evaporated and the residue was hydrolysed in N-HCl for 1hr. at 100° and neutralized with an excess of Amberlite IR-45 (OH^- form) resin. The hydrolysate was passed through a column containing Dowex 1 (borate form) resin (50ml.), the column was washed with water and elution was carried out with 0·5N-boric acid solution. The eluate was collected in fractions, which were examined chromatographically for the presence of 3-*O*-methyl-D-mannose. Appropriate fractions were combined and evaporated repeatedly with methanol to remove boric acid. Attempts to crystallize the sugar were unsuccessful.

The syrupy 3-*O*-methyl-D-mannose (150mg.) was dissolved in water (10ml.) and treated with sodium borohydride (100mg.) in water (5ml.). The reaction mixture was kept at room temperature for 16hr., and then an excess of Amberlite IR-120 (H^+ form) resin was added. The mixture was stirred for 15min. and filtered. The filtrate was repeatedly evaporated with methanol to remove boric acid, and 3-*O*-methyl-D-mannitol was obtained from the residue by crystallization from butan-1-ol. The yield was 50mg., m.p. 133–134°, $[\alpha]_D^{26}$ +17·1±1° (c 0·55 in water) (Bladon & Owen, 1950, give m.p. 132–133°, $[\alpha]_D^{20}$ +16·5°).

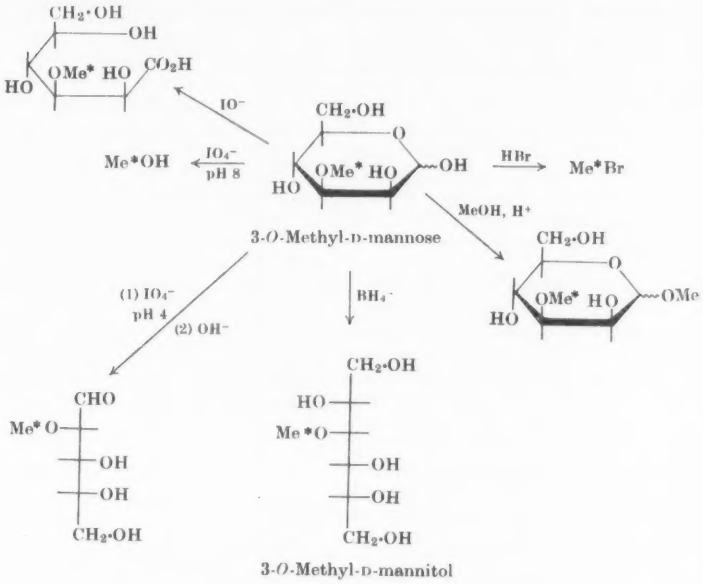
RESULTS

Preliminary experiments. Since very small quantities of the labelled compound were available (the amounts used for each experiment were too small to detect chemically), the reactions were followed only by the observations of radioactive products (either by counting different fractions or by examining radioactivity on paper chromatograms of reaction mixtures). These preliminary experiments are summarized in Table 1 and illustrated in Scheme 1.

Identification of 3-O-*methyl*-D-*mannitol*. A sample of the labelled sugar was reduced with sodium borohydride to give a product (see Table 1, Expt. 6), chromatographically and electrophoretically identical with the synthetic 3-*O*-methyl-D-mannitol.

Table 1. *Summary of experiments carried out on the labelled reducing sugar*

Expt. no.	Treatment	Result
1	Solution treated with Dowex 50 (H^+ form)	^{14}C not adsorbed on resin
2	Kuhn–Roth oxidation (Milton & Waters, 1955)	No [^{14}C]acetic acid produced
3	Sugar treated with HBr (Hough, Jones & Wadman, 1950)	[^{14}C]Methyl bromide formed
4	Paper chromatography:	$R_{Rhamnose}$
		Sugar / 3-*O*-Methylglucose
	Solvent 1	1·02 / —
	Solvent 2	0·94 / 0·88
	Solvent 3	0·99 / 0·93
5	Paper electrophoresis in 0·05M-sodium tetraborate	M_{Glc} 0·48
6	Reduced with sodium borohydride	Product chromatographically different from starting material
7	Heated at 100° with methanol containing 3% (w/v) of HCl	Product chromatographically and electrophoretically different from starting material
8	Oxidized with hypoiodite (Willstätter & Schudel, 1918)	Product chromatographically different from starting material
9	Oxidized with periodate at pH8 (Hough & Perry, 1956)	[^{14}C]Methanol produced
10	Oxidized with periodate at pH4 followed by mild alkali hydrolysis (Huffman, Lewis, Smith & Spriesterbach, 1955)	Chromatography and electrophoresis showed same product (2-*O*-methylarabinose) given by similar treatment of 3-*O*-methylglucose
11	Periodate oxidation of methanolysis product (see Expt. 7 above) at pH4	No oxidation



Scheme 1. Reactions of 3-*O*-methyl-D-mannose.

The product was mixed with synthetic 3-*O*-methyl-D-mannitol and the mixture was recrystallized from butan-1-ol containing 3% (v/v) of water. It is shown below that the added radioactive material crystallized with the authentic 3-*O*-methyl-D-mannitol, and that the specific activity remained constant through six recrystallizations.

Examination of the 'neutral fraction'. The 'neutral fraction' slowly diffused through a dialysis sac (Visking). It was somewhat retarded by Sephadex G-25, the radioactive material appearing in 1·1–1·5 times the void volume. These results suggest that the compound might possess a molecular weight of 1000–5000. Paper chromatography in propan-1-ol–aq. ammonia (sp.gr. 0·88)–water (6:3:1, by vol.) gave a fairly broad band with an R_F similar to that for a maltodextrin of chain length 7. Examination of the products of partial hydrolysis (0·1N-hydrochloric acid for 1hr. at 100°) of the 'neutral fraction' by paper chromatography in solvent 1 showed a series of radioactive products with ascending R_F values up to and including that corresponding to the 3-*O*-methylmannose. This suggests that the radioactive component of the 'neutral fraction' is an oligosaccharide or small polysaccharide, or possibly a series of these covering a range of molecular weights. However, paper chromatography in a variety of solvents was not entirely consistent with this view and it is not yet possible to classify this compound with confidence.

DISCUSSION

Although the nature of the compound in *S. griseus* containing a 3-*O*-methylmannose residue has not been studied in detail, the methylated sugar itself has been fully characterized. Evidence for the identity of the reducing sugar as 3-*O*-methyl-D-mannose is presented in Table 1 and below, and the reactions are illustrated in Scheme 1. The result of Expt. 3 (Table 1) suggests that the labelled atom might be present in an *O*-methyl group. The carbohydrate nature of the compound is likely from its susceptibility to oxidation with periodate, its chromatographic properties and its migration on electrophoresis in borate. Evidence that it is a reducing sugar was provided by the results of Expt. 6 (reduction by borohydride) and Expt. 7 (methyl glycoside formation). The results of Expt. 8 (hypoiodite oxidation) suggest that it is an aldose.

Treatment of 3-*O*-methyl-D-glucose with periodate at pH 4 (Expt. 10) oxidizes the sugar between C-1 and C-2; the product, 4-*O*-formyl-2-*O*-methyl-D-arabinose, is not further attacked, provided that the formyl ester group remains intact (Barker &

Smith, 1952). Mild hydrolysis in alkali removes the formyl group to give 2-*O*-methyl-D-arabinose (Huffman *et al.* 1955). When the radioactive sugar was treated in the same way as the 3-*O*-methyl-D-glucose a chromatographically identical product was obtained. Since the sugar is an aldose and yet is not identical with 3-*O*-methylglucose (Expt. 4), it must be the epimeric sugar 3-*O*-methylmannose. The result of Expt. 11 agrees with this, since a methyl mono-*O*-methylhexopyranoside with the ether group at any position other than at C-3 would be oxidized by periodate.

Proof of identity was obtained by co-crystallization of the reduced labelled sugar with synthetic 3-*O*-methyl-D-mannitol. For one to six recrystallizations the specific activities were respectively 686, 684, 690, 684, 680 and 686 counts/min./mg. The identification of the sugar, its derivatives and degradation products was achieved with radioactivity as the only means of detection. There were two reasons for adopting this procedure: (*a*) only very small quantities of material were available, and it was not possible to detect either starting material or products by chemical methods; (*b*) although the labelled reducing sugar was radiochemically pure (shown by chromatography, electrophoresis and behaviour in the various chemical reactions applied to it), it was not known whether it was chemically pure. Since such small amounts were available it would have been difficult to establish chemical purity.

Naturally occurring *O*-methyl sugars, although not common, have been isolated from a number of different sources. Thus certain antibiotics produced by Actinomycetes contain sugar residues bearing *O*-methyl groups. One example is the sugar noviose (Walton, Rodin, Stammer, Holly & Folkers, 1956; Hinman, Caron & Hoeksema, 1957). Cardiac glycosides frequently contain such residues, an example being 6-deoxy-3-*O*-methyl-L-mannose (Muhr, Hunger & Reichstein, 1954; see also Pigman, 1957), and they have been identified among the products of mycobacteria (Lee & Ballou, 1964). As far as we know, this is the first identification of 3-*O*-methyl-D-mannose from natural sources. Presumably, biosynthesis occurs by methyl group transfer from L-methionine to a sugar or sugar derivative. Such a process has also been demonstrated in the biosynthesis of noviose (Birch, Cameron, Holloway & Rickards, 1960).

The preparation of 3-*O*-methyl-D-mannose from D-mannose involves first protecting the hydroxyl groups at positions 1, 4 and 6, followed by partial methylation. Since methyl 4,6-*O*-benzylidene-α-D-mannoside is in the C-1 conformation (Reeves, 1949; Aspinall & Zweifel, 1957), the hydroxyl group at C-3 is equatorial and that at C-2 is axial. The equatorial hydroxyl group would be expected to be more readily methylated than the axial group, and partial methylation would produce a higher proportion of the 3-*O*-methyl derivative than of the 2-*O*-methyl derivative. Oxidation of the partially methylated methyl mannosides with periodate destroys methyl 2-*O*-methyl-D-mannoside and methyl D-mannoside, but methyl 3-*O*-methyl-D-mannoside is unaffected. 3-*O*-Methyl-D-mannose could be separated from 2,3-di-*O*-methyl-D-mannose and other impurities by chromatography on a borate column.

We thank Glaxo Ltd. for a grant and for cultures of *Streptomyces griseus*. Grants are also acknowledged from the Science Research Council and U.S. Department of Public Health (AI-05237-01 BIO).

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Biochemical Studies of Toxic Agents

METABOLIC RING-FISSION OF *CIS*- AND *TRANS*-ACENAPHTHENE-1,2-DIOL

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(*Received* 3 *June* 1965)

1. The metabolism of *cis*- and *trans*-acenaphthene-1,2-diol has been studied after the administration of these compounds to rats by subcutaneous injection and by stomach tube. 2. 1,8-Naphthalic acid has been isolated as its anhydride from the urine of the dosed animals. 3. A spectrophotometric method for the determination of free and conjugated 1,8-naphthalic acid in urine has been developed and has been used in the study of the metabolism of the acenaphthene-1,2-diols. 4. The urine of rats dosed with *cis*-acenaphthene-1,2-diol by subcutaneous injection was shown by paper chromatography to contain both *cis*- and *trans*-acenaphthene-1,2-diol. Similar findings were obtained after the subcutaneous injection of *trans*-acenaphthene-1,2-diol.

Acenaphthylene is one of the few carbocyclic compounds that have been shown to undergo ring-fission in the animal body, for Hopkins, Brooks & Young (1962) were able to isolate 1,8-naphthalic acid, as its anhydride, from the urine of rats and rabbits after the administration of this hydrocarbon. In addition, they isolated *cis*- and *trans*-acenaphthene-1,2-diol from the urine of the dosed animals, and, in view of the suggestion (Young, 1950) that diols might undergo ring-fission *in vivo* to yield dicarboxylic acids, the possibility that the diols formed from acenaphthylene can give rise to 1,8-naphthalic acid in the animal body has now been investigated.

MATERIALS

All melting points are uncorrected. The melting point of naphthalic anhydride was determined by heating the compound either on a brass block or in a bath containing silicone fluid (MS550; Hopkin and Williams Ltd., Chadwell Heath, Essex). Elementary microanalyses were carried out by Weiler and Strauss, Oxford. The Ring Index numbering of compounds (Patterson, Capell & Walker, 1960) has been used throughout this paper.

cis- *and* trans-*Acenaphthene*-1,2-*diol*. These compounds were prepared by the methods described by Hopkins *et al.* (1962) in which the Prévost (1933*a*,*b*) reaction was used. Acenaphthylene was treated with silver benzoate at room temperature to give the monobenzoate of *cis*-acenaphthene-1,2-diol. When the reaction was repeated at 80° the dibenzoate of *trans*-acenaphthene-1,2-diol was obtained. The diols were liberated by hydrolysis of the benzoates with methanolic KOH, and after recrystallization *cis*-acenaphthene-1,2-diol was obtained as needles, m.p. 207–208°, and (±)-*trans*-acenaphthene-1,2-diol as plates, m.p. 157–158°.

The synthetic diols used in this work were carefully purified, and each was tested for the presence of the other by paper-chromatographic methods, details of which are given below. With solvent mixtures *A* and *B* (see the Methods section) it was possible to detect *trans*-diol when 40μg. of a mixture containing 99·5% of *cis*-acenaphthene-1,2-diol and 0·5% of *trans*-acenaphthene-1,2-diol was chromatographed. When 40μg. of the synthetic *cis*-acenaphthene-1,2-diol was chromatographed under the same conditions, no *trans*-diol was detected. By the use of solvent mixtures *C* and *D* (see the Methods section) it was possible to detect the *cis*-diol in a mixture of 99·8% of *trans*-diol and 0·2% of *cis*-diol when 100μg. of the mixture was chromatographed. No *cis*-diol was detected when 100μg. of synthetic *trans*-acenaphthene-1,2-diol was chromatographed. It is therefore apparent that the *cis*-diol contained less than 0·5% of the *trans*-diol, and that the *trans*-diol contained less than 0·2% of the *cis*-diol.

1,8-*Naphthalic anhydride*. Acenaphthene was oxidized with $Na_2Cr_2O_7$ in acetic acid according to the method of Graebe & Gfeller (1892), and naphthalic anhydride was separated from the reaction mixture and purified as described by Hopkins *et al.* (1962). The product was obtained as colourless needles, m.p. 273–274°, which yielded *N*-anilinonaphthalimide, m.p. 215–216°, by interaction with phenylhydrazine (Hopkins *et al.* 1962).

Naphthalic anhydride was also prepared by the oxidation of *cis*- and *trans*-acenaphthene-1,2-diol with alkaline $KMnO_4$. Diol (0·05g.) was dissolved by heating in 200ml. of 2N-NaOH, and the solution was then cooled to room temperature. Aq. 1% (w/v) $KMnO_4$ was added slowly with stirring until a small excess was present. After the reaction mixture had stood at room temperature for 20min., the excess of $KMnO_4$ was removed by the addition of $Na_2S_2O_5$. The precipitate was removed by centrifuging, and was washed once with 200ml. of water. The washing and main supernatant solution were combined, brought to pH2 with conc. HCl, left at room temperature overnight and then extracted twice with equal volumes of ether. The

combined ether extracts were evaporated to dryness under reduced pressure, and the residue from each preparation was crystallized from aqueous acetone. Both preparations gave pale-yellow needles, m.p. 269–270°. The melting point was not depressed on admixture with naphthalic anhydride prepared by oxidation of acenaphthene with $Na_2Cr_2O_7$ in acetic acid, and the products prepared by the two methods gave the same infrared spectrum. By oxidation with alkaline $KMnO_4$, naphthalic anhydride was obtained in 76% yield from *cis*-acenaphthene-1,2-diol and in 84% yield from the *trans*-diol.

Disodium 1,8-naphthalate. This compound was prepared by treating naphthalic anhydride with 95% of the theoretical amount of NaOH necessary to form the disodium salt, and then removing the excess of naphthalic anhydride. To 5g. of naphthalic anhydride was added 1·92g. of NaOH in 250ml. of water. The mixture was heated under reflux in a boiling-water bath for 1hr., during which time nearly all the anhydride dissolved. The suspension was cooled and filtered to remove naphthalic anhydride, and the disodium salt of 1,8-naphthalic acid was precipitated from the filtrate by the addition of acetone. The disodium salt was separated and washed with acetone, and was obtained as a white solid (5·50g.), readily soluble in water. This product lost no weight when heated at 110°.

Acenaphthen-1-one. This compound was obtained as prisms, m.p. 119–120·5°, by the acid decomposition of *trans*-acenaphthene-1,2-diol as described by Hopkins *et al.* (1962).

METHODS

Animals and dosing. Male rats (body wt. 150–200g.) were used. They were of the Wistar strain or a black-hooded strain, and were housed in metabolism cages from which the urine was collected separately from the faeces. They were fed on rat cakes [J. Murray and Sons (London) Ltd.] and had access to water at all times.

In some experiments the acenaphthene-1,2-diol was administered by stomach tube and in others by subcutaneous injection. Each time a rat was given a diol by stomach tube it received 1·25ml. of a 20% (w/v) suspension of the diol in 1% (w/v) starch solution. When a diol was administered by subcutaneous injection each dose consisted of 1·0ml. of a 20% (w/v) suspension of the diol in arachis oil.

Urine was collected daily throughout the period of dosing and for a definite time after the last dose. The urine was filtered through glass wool and stored in the refrigerator.

Paper chromatography. cis- and *trans*-Acenaphthene-1,2-diol and 1,8-naphthalic anhydride were detected on paper chromatograms as fluorescent spots under ultraviolet light, after the chromatograms had been sprayed with ethanol–conc. H_2SO_4–water (18:1:1, by vol.) and heated (Hopkins *et al.* 1962). Four different solvent systems were employed, the compositions of which were: *A*, methanol–carbon tetrachloride (9:1, v/v); *B*, methanol–cyclohexane (9:1, v/v); *C*, benzene–carbon tetrachloride–water–aq. NH_3 (sp.gr. 0·88) (9:1:8:2, by vol.); *D*, benzene–carbon tetrachloride–water–acetic acid (9:1:8:2, by vol.). The components of systems *C* and *D* were shaken vigorously for 1–2min. and then allowed to separate for 30min. The upper layer of each was used as the mobile phase, and the lower layer as the stationary phase. Papers were equilibrated overnight in an atmosphere of the stationary phase. Whatman no. 1 paper was used, with the ascending method for systems *A* and *B*, and the descending method for systems *C* and *D*. The minimum quantity of either diol that could be detected was 0·2μg. Typical R_F values for *cis-* and *trans*-acenaphthene-1,2-diol in the four solvent systems are shown in Table 1.

Table 1. *Paper chromatography of* cis- *and* trans-*acenaphthene-1,2-diol*

Experimental details are given in the text.

Solvent system	R_F	
	cis-Diol	*trans*-Diol
A	0·69	0·77
B	0·70	0·77
C	0·53	0·23
D	0·62	0·25

Isolation of 1,8-naphthalic acid. This compound was isolated in the form of its anhydride from the acidified urine of dosed animals after the urine had been extracted under neutral conditions with ether to remove any free *cis-* or *trans*-acenaphthene-1,2-diol. The urine was adjusted to pH 2 with conc. HCl and was then shaken with four separate portions of ether, each equal in volume to the urine. The combined ether extracts were evaporated to dryness and the residue so obtained was stirred with a small volume of ice-cold ethanol. The material that did not dissolve in the ethanol was separated and dissolved in warm 2N-NaOH. The alkaline solution was decolorized with charcoal and filtered. The precipitate that formed on acidifying the filtrate was crystallized from aqueous acetone. In some cases the product was recrystallized from hot conc. HNO_3 to which had been added one-tenth its volume of water, a procedure that had been shown to be advantageous in experiments with synthetic naphthalic anhydride. To ensure that the final product was in the anhydride form it was heated to constant weight at 110°.

Isolation of cis- *and* trans-*acenaphthene-1,2-diol.* These compounds were isolated from the urine of dosed animals, either before or after it had been treated with a rat-liver preparation containing β-glucuronidase, as described by Hopkins *et al.* (1962).

Determination of free and total naphthalic acid in urine. These determinations were based on the extinction shown by naphthalic anhydride at 339mμ. To determine free naphthalic acid the urine was clarified by centrifuging and it was then diluted with one-third its volume of water. To 1ml. of the diluted urine was added 5ml. of 0·45% $ZnSO_4$ solution followed by 3ml. of 0·1N-NaOH. The suspension that formed was mixed well and after 3min. it was centrifuged. The precipitate was washed once with 5ml. of M-$NaHCO_3$ and the washing was added to the supernatant solution, which was then brought to pH7 with 2N-HCl and washed twice by shaking with one-fifth its volume of ether to remove any acenaphthen-1-one that might have been present. The aqueous solution was then brought to pH 2 with conc. HCl and after 1hr. at room temperature it was extracted by shaking six times with equal volumes of ether. The combined ether extracts were evaporated to dryness under reduced pressure. The residue obtained was heated at 110° for 1hr. and then dissolved in a known volume of butan-1-ol (analytical

grade) to give a solution containing about 15μg. of naphthalic anhydride/ml. After any undissolved matter had been removed by centrifuging, the concentration of naphthalic anhydride was determined by measuring the extinction of the butan-1-ol solution at 339mμ against a blank solution prepared by carrying out the procedures just described on urine from undosed rats. When the extinctions of solutions of naphthalic anhydride in butan-1-ol (5–25μg./ml.) were measured with a Hilger Uvispek spectrophotometer at 339mμ they varied linearly with the concentration of the anhydride. In experiments in which the method was tested by adding the disodium salt of 1,8-naphthalic acid to normal rat urine in amounts equivalent to 0·1, 0·2, 0·3, 0·4 and 0·5mg. of naphthalic anhydride/ml. the recoveries were 87, 101, 96, 98 and 99% respectively.

Total naphthalic acid was determined after acid hydrolysis. The urine was diluted with one-third its volume of water, and 1ml. of the diluted urine was heated under reflux in a boiling-water bath with 0·5ml. of 50% (v/v) H_2SO_4 for 4hr., conditions that had been found to give maximal liberation of naphthalic acid from its conjugates in urine. The hydrolysed urine was adjusted to pH8–9 with 40% (w/v) NaOH solution and allowed to stand overnight. Then 16ml. of 0·45% $ZnSO_4$ solution and 9·6ml. of 0·1N-NaOH were added and the precipitate was washed with 16ml. of M-$NaHCO_3$, and thereafter the procedure was as described above for the determination of free naphthalic acid.

When two rats were each injected subcutaneously with 1ml. of an aqueous solution containing 0·05g. of the disodium salt of 1,8-naphthalic acid, analysis of the urine collected during the next 4 days showed that the free naphthalic acid excreted during this period corresponded to 70% of that administered. The total naphthalic acid found after periods of hydrolysis of 1, 4, 6 and 8hr. corresponded to 77, 79, 79 and 78% respectively of the naphthalic acid administered. Naphthalic acid was stable under these conditions, for when normal rat urine to which 400μg. of disodium naphthalate had been added/ml. was hydrolysed for 8hr. recoveries of 98 and 99% were obtained.

RESULTS

Metabolism of cis-*acenaphthene*-1,2-*diol*

Isolation of 1,8-*naphthalic anhydride.* When a total of 7·2g. of *cis*-acenaphthene-1,2-diol was administered to 18 rats by subcutaneous injection, 0·147g. of naphthalic anhydride was separated from the urine of the dosed animals, m.p. 271–272°, mixed m.p. 271–272° (Found: C, 72·8; H, 3·2. Calc. for $C_{12}H_6O_3$: C, 72·7; H, 3·1%). By reaction with phenylhydrazine the compound from urine gave a product, m.p. 213–215°, and m.p. 214–215° when mixed with synthetic *N*-anilinonaphthalimide (m.p. 215–216°) (Found: C, 74·5; H, 4·2; N, 9·9. Calc. for $C_{18}H_{12}N_2O_2$: C, 75·0; H, 4·2; N, 9·7%). The ultraviolet and infrared spectra of the compound from urine and its behaviour on paper chromatograms corresponded to those of synthetic 1,8-naphthalic anhydride.

From the urine of 12 rats that had received a total of 6g. of *cis*-acenaphthene-1,2-diol by stomach tube, 0·106g. of naphthalic anhydride was obtained, m.p. 272–273°, mixed m.p. 272–273° (Found: C, 72·2; H, 3·2%).

Quantitative studies. Two black-hooded rats were injected subcutaneously with 0·093g. (0·5m-mole) of *cis*-acenaphthene-1,2-diol in 1ml. of arachis oil. By the use of the analytical procedure described above it was found that the excretion of naphthalic acid was virtually complete in the first 4 days after injecting the diol and that in this time the amounts of free and combined naphthalic acid in the urine corresponded to 9·2 and 0·4% respectively of the diol administered.

Paper-chromatographic studies. Two male black-hooded rats were each given by subcutaneous injection 0·2g. of *cis*-acenaphthene-1,2-diol in 1ml. of arachis oil, and two control rats were each injected with 1ml. of arachis oil. The urine from each pair of animals was collected for 24hr. and was then extracted continuously with ether for 6hr., after which the volume of the ether extract was adjusted to 100ml. The ether solution was shaken with 10ml. of 2N-sodium hydroxide to remove naphthalic acid and was then concentrated to 10ml. A third ether solution was prepared in the same way from a solution of a mixture of 5mg. of *cis*- and 5mg. of *trans*-acenaphthene-1,2-diol in 50ml. of normal rat urine. All three ether solutions were examined simultaneously by paper chromatography with solvent mixtures *A* and *B*. No diols were found in the extract of the urine from the control animals, but the extract of the urine from the animals dosed with *cis*-acenaphthene-1,2-diol showed the same results as were obtained with the extract of the reference urine containing *cis*- and *trans*-acenaphthene-1,2-diol.

After the free diols had been removed from the urine by ether extraction each urine was examined for the presence of conjugated diols. Dissolved ether was removed by warming the urine, after which the urine was incubated at pH5·2 with a rat-liver preparation containing β-glucuronidase as described by Hopkins *et al.* (1962). The urine was then brought to pH7–8 and examined, as described above, for the presence of free diols by using the reference solution obtained by ether extraction of normal rat urine to which *cis*- and *trans*-acenaphthene-1,2-diol had been added. The chromatograms were similar to those described above, and showed that when it was treated with rat-liver preparation the urine of rats dosed with *cis*-acenaphthene-1,2-diol yielded further amounts of the *cis*- and *trans*-forms of the diol.

Isolation of cis-*diol from urine.* When the urine of 16 rats that had received a total of 6·4g. of *cis*-acenaphthene-1,2-diol by subcutaneous injection was incubated with a rat-liver preparation containing β-glucuronidase it yielded 0·061g. of *cis*-diol,

m.p. and mixed m.p. 207–208° (Found: C, 77·2; H, 5·2. Calc. for $C_{12}H_{10}O_2$: C, 77·4; H, 5·4%). This material gave a diacetate, m.p. and mixed m.p. 130–131° (Found: C, 70·8; H, 5·3. Calc. for $C_{16}H_{14}O_4$: C, 71·1; H, 5·2%). In a similar experiment 0·159g. of *cis*-acenaphthene-1,2-diol, m.p. and mixed m.p. 206–207°, was isolated from the urine of 12 rats that had received a total of 6g. of the diol by stomach tube.

Attempts to isolate *trans*-acenaphthene-1,2-diol from the urine of rats that had been dosed with *cis*-acenaphthene-1,2-diol, either by subcutaneous injection or by stomach tube, were all unsuccessful, irrespective of whether the urine had been incubated with a rat-liver β-glucuronidase preparation.

Metabolism of trans-*acenaphthene*-1,2-*diol*

Isolation of 1,8-*naphthalic anhydride.* In an experiment in which 7·2g. of (±)-*trans*-acenaphthene-1,2-diol was administered to 18 rats by subcutaneous injection, 0·215g. of naphthalic anhydride, m.p. and mixed m.p. 270–271°, was separated from the urine of the dosed animals (Found: C, 72·6; H, 2·6%). This compound showed the same ultraviolet and infrared spectra and R_F values as synthetic naphthalic anhydride, and by reaction with phenylhydrazine it yielded *N*-anilinonaphthalimide, m.p. and mixed m.p. 214–215° (Found: C, 74·7; H, 4·3; N, 9·7%).

From the urine of rats that had received a total of 6·0g. of (±)-*trans*-acenaphthene-1,2-diol by stomach tube, 0·090g. of naphthalic anhydride, m.p. and mixed m.p. 271–272°, was obtained (Found: C, 73·1; H, 3·4%).

Quantitative studies. Two male black-hooded rats were injected subcutaneously with 0·093g. (0·5m-mole) of (±)-*trans*-acenaphthene-1,2-diol in 1ml. of arachis oil. Analysis of the urine collected in the next 4 days showed the presence of free and conjugated 1,8-naphthalic acid in amounts corresponding to 9·6 and 1·4% respectively of the diol administered. No naphthalic acid was detected in the urine excreted on the fifth and sixth days after injecting the diol.

Paper-chromatographic studies. The urine of rats that had each received a single dose of 0·2g. of (±)-*trans*-acenaphthene-1,2-diol by subcutaneous injection was examined by the procedures described above for experiments in which the *cis*-diol was administered. Both before and after hydrolysis with the rat-liver β-glucuronidase preparation, it was possible to detect *cis*- and *trans*-acenaphthene-1,2-diol in the urine of the dosed animals.

Isolation of trans-*diol from urine.* From the enzyme-hydrolysed urine of 16 rats that had received a total of 6·4g. of (±)-*trans*-acenaphthene-1,2-diol by subcutaneous injection was isolated 0·049g. of mixed (+)- and (±)-*trans*-diol, m.p. 142–147°, $[\alpha]_{5461}^{20}+23°$ (*c* 0·065 in benzene) (Found: C, 77·1; H, 5·6). Under the same conditions the urine of 12 rats that had received a total of 6·0g. of (±)-*trans*-acenaphthene-1,2-diol by stomach tube yielded 0·119g. of mixed (+)- and (±)-*trans*-diol, m.p. 144–147°, $[\alpha]_{5461}^{25}+49°$ (*c* 0·062 in benzene). It is noteworthy that the melting points and specific rotations of the isolated *trans*-diols resemble those obtained for the mixed (+)- and (±)-*trans*-acenaphthene-1,2-diol isolated after the administration of acenaphthylene to rats (Hopkins *et al.* 1962).

After enzymic hydrolysis the urine of the rats that had been dosed with a total of 6·4g. of (±)-*trans*-acenaphthene-1,2-diol by subcutaneous injection also yielded *cis*-acenaphthene-1,2-diol. The isolated diol weighed 0·036g., and its m.p., 206–207°, was not depressed on admixture with synthetic *cis*-acenaphthene-1,2-diol. It gave a diacetate, m.p. and mixed m.p. 130–131°.

Stability of cis- *and* trans-*acenaphthene*-1,2-*diol in rat urine*

The finding that 1,8-naphthalic acid can be separated as its anhydride from the urine of rats after the administration of *cis*- and *trans*-acenaphthene-1,2-diol made it necessary to determine whether any 1,8-naphthalic acid was formed in the urine, or during the isolation process, by breakdown of acenaphthenediols. This was tested by taking two 500ml. portions of normal rat urine and adding 0·425g. of *cis*-acenaphthene-1,2-diol to one and 0·425g. of the *trans*-isomer to the other. Each urine was saturated with diol by repeated gentle warming followed by mechanical shaking. The urines were then kept at 37° for 24hr. and each was then subjected to the treatment used to isolate 1,8-naphthalic acid. None was found.

The fact that *cis*-acenaphthene-1,2-diol was found in the urine of rats dosed with its *trans*-isomer and vice versa made it necessary to determine whether this was the outcome of an interconversion of the free diols occurring in the urine itself, and whether interconversion could be brought about by the liver preparation used to hydrolyse diol conjugates. Fresh normal rat urine was adjusted to pH 7–8 and in three 110ml. portions of the urine the following were dissolved: (1) 8mg. of *cis*-acenaphthene-1,2-diol; (2) 0·04mg. of *trans*-acenaphthene-1,2-diol; (3) 8mg. of *cis*-diol and 0·04mg. of *trans*-diol. The three urines were left at room temperature for 24hr. and each was then extracted by mechanical shaking for four 30min. periods with equal volumes of ether. The ether extracts from each urine were combined and evaporated under reduced pressure to 10ml. These solutions were examined by paper chroma-

tography with 100μl. of each solution and solvent mixtures *A* and *B*. The chromatogram from the extract of (1) showed no trace of *trans*-diol, whereas those from the extracts of (2) and (3) showed that *trans*-diol, alone and when mixed with 200 times as much *cis*-diol, can be readily detected by the procedure used.

Another experiment was carried out that was essentially the same as that just described except that the additions of diols were as follows: (1) 8mg. of *trans*-acenaphthene-1,2-diol; (2) 0·04mg. of *cis*-acenaphthene-1,2-diol; (3) 8mg. of *trans*-diol and 0·04mg. of *cis*-diol. When the ether extracts were chromatographed with solvent mixtures *C* and *D*, no *cis*-diol was found on the chromatogram of the extract of (1). The extracts of (2) and (3) showed that *cis*-diol, alone and when mixed with 200 times its weight of *trans*-diol, can easily be detected. It follows from these experiments that, if interconversion of the free acenaphthenediols occurs on standing in urine for 24hr., this takes place to the extent of less than 0·5%.

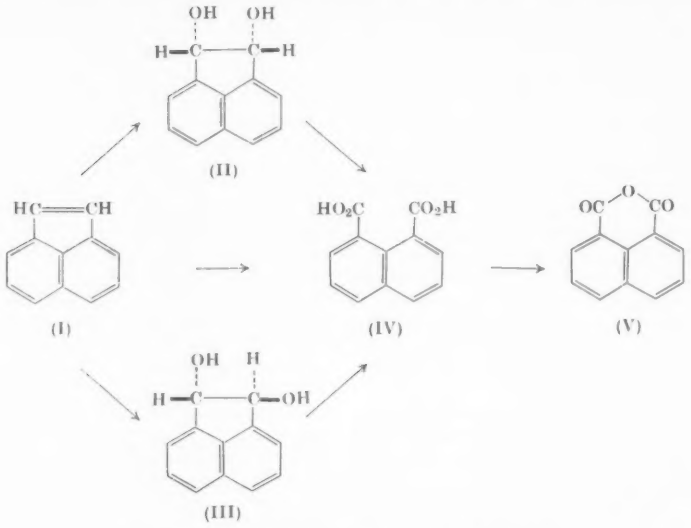
The possible effect of the rat-liver β-glucuronidase preparation on the interconversion of the free acenaphthenediols was tested in the following experiment. A solution of 16mg. of *cis*-acenaphthene-1,2-diol in 120ml. of water was prepared as well as another 120ml. of aqueous solution containing the same amount of the *trans*-isomer. The solutions were incubated at 37° for 24hr. with 0·2M-acetate buffer, pH 5·2, together with the liver preparation obtained as described by Hopkins *et al.* (1962). At the end of the incubation the suspensions were centrifuged, adjusted to pH 7–8 and extracted by mechanical shaking with four equal volumes of ether. The combined ether extracts from each solution were washed with 2N-sodium hydroxide to remove acids and, after being concentrated under reduced pressure, were examined by ascending paper chromatography with solvent mixture *B*. The results showed that, if interconversion of the free *cis*- and *trans*-diols occurred in the presence of the liver preparation, it took place to the extent of less than 0·5%. The experiments, however, do not exclude the possibility that interconversion of the diols resulted from hydrolysis of their conjugates (cf. Brooks & Young, 1956).

DISCUSSION

It has been suggested that dihydrodiols might serve as intermediates in the oxidative metabolism of cyclic hydrocarbons and that dicarboxylic acids might be among the products to which they give rise (Young, 1950; Hopkins & Young, 1961). Various hydrocarbons, e.g. naphthalene (Young, 1947; Booth & Boyland, 1949), anthracene (Boyland & Levi, 1935), phenanthrene (Young, 1947; Boyland & Wolf, 1950; Boyland & Sims, 1962) and indene (Brooks & Young, 1956), give rise to dihydrodiols in animals, but none of these hydrocarbons or the dihydrodiols has so far been reported to undergo ring-fission in the animal body. Benzene is known to be metabolized to muconic acid in animals (Jaffé, 1909; Bernhard & Gressly, 1941), and evidence has been obtained for its conversion *in vivo* into a dihydrodiol (Sato, Fukuyama, Suzuki & Yoshikawa, 1963). The dihydrodiol so formed, cyclohexa-1,3-diene-5,6-diol, has been synthesized (Nakajima, Tomida, Hashizume & Takei, 1956), but Tomida & Nakajima (1960) obtained no evidence for its conversion into muconic acid when they administered it to rats. Since Hopkins *et al.* (1962) showed that acenaphthylene (I) is converted in the rat into 1,8-naphthalic acid (IV) and into *cis*- and *trans*-acenaphthene-1,2-diol (II and III) and thereby undergoes ring-fission as well as forming dihydrodiols (see Scheme 1), it was decided to determine whether these dihydrodiols are metabolized in the rat to 1,8-naphthalic acid. Preliminary observations were made that indicated that this occurs (Hopkins & Young, 1961), and these have been confirmed and extended in the present work, in which *cis*- and *trans*-acenaphthene-1,2-diol were administered to rats by subcutaneous injection or by stomach tube and in every case 1,8-naphthalic acid was separated in the form of its anhydride (V) from the urine of the dosed animals.

The question arises whether the 1,8-naphthalic acid present in the urine was formed by a metabolic reaction within the tissues or whether it originated in some other way. It was shown that *cis*- and *trans*-acenaphthene-1,2-diol give 1,8-naphthalic acid when oxidized at room temperature by potassium permanganate in alkaline solution, but that in the absence of oxidant they are stable at the pH of rat urine. It was also shown that when *cis*- or *trans*-acenaphthene-1,2-diol was incubated at 37° for 24hr. in rat urine no 1,8-naphthalic anhydride was found when the urine was subjected to the treatment used to obtain this compound from the urine of rats that had been dosed with *cis*- or *trans*-acenaphthene-1,2-diol.

Although the formation of acids by the fission of diphenolic compounds by micro-organisms is well established (cf. Ornston & Stanier, 1964), the fission of dihydrodiols by micro-organisms does not appear to have been demonstrated. Nevertheless the possibility has to be considered that the conversion of the dihydrodiols into 1,8-naphthalic acid may occur as a result of microbial activity. The experiments just described, however, make it appear most unlikely that such a reaction occurred in the urine of the rats that had been dosed with *cis*- and *trans*-acenaphthene-1,2-diol. There remains



Scheme 1. Metabolic formation of 1,8-naphthalic acid.

the possibility that formation of 1,8-naphthalic acid was a result of the action of micro-organisms in the intestinal tract. Although the dihydrodiols were administered by subcutaneous injection this does not exclude the possibility that they were carried in the bile into the intestine and were there acted on by micro-organisms to give 1,8-naphthalic acid, which was absorbed and excreted in the urine.

Several groups of workers have demonstrated the occurrence in mammalian tissues of enzyme systems that bring about ring-fission. For example, Douglass, Shook, Weir & Hogan (1959) have shown the cleavage of gentisic acid by an enzyme system in rat liver, and Knox & Edwards (1955) have described an enzyme preparation from rat and guinea-pig liver that brings about ring-fission of homogentisic acid. The oxidation of *cis*- and *trans*-acenaphthene-1,2-diol to 1,8-naphthalic acid by rat tissues is at present under investigation in the authors' Laboratory, and microsomal preparations from liver have been shown to bring about this reaction. There is therefore little doubt that rat liver itself can bring about splitting of the five-membered ring of *cis*- and *trans*-acenaphthene-1,2-diol with the formation of 1,8-naphthalic acid.

The authors acknowledge the support this work has received from the Endowment Fund of St Thomas's Hospital and the Central Research Fund of the University of London.

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The Incorporation of [1-^{14}C]Acetate into the Methyl Ketones that Occur in Steam-Distillates of Bovine Milk Fat

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(*Received* 14 *June* 1965)

1. The ^{14}C-labelling of the fatty acids and the methyl ketones in steam-distillates of milk fat from a lactating cow that had been injected intravenously with [1-^{14}C]acetate was determined. 2. The labelling patterns of the C_6–C_{16} fatty acids and the corresponding methyl ketones with one fewer carbon atoms were similar, particularly so for the C_5–C_{10} compounds at 9 and 22 hr. after the injection of [1-^{14}C]acetate. The isolation of ^{14}C-labelled methyl ketones in the range C_3–C_{15} is evidence that the β-oxo acid precursors, which are glyceride-bound in the milk fat, are synthesized in the mammary gland from acetate. The absence of heptadecan-2-one in steam-distillates and the extremely low specific radioactivity of stearic acid are further evidence for this biosynthetic pathway. 3. The specific radioactivities of the C_5–C_{15} methyl ketones were higher (with the exception of C_9 methyl ketone in the second milking) than the specific activities of the corresponding fatty acids with one more carbon atom. This is consistent with the methyl ketone precursors' being formed during the biosynthesis of fatty acids rather than being products of β-oxidation of fatty acids.

When milk fat is heated in the presence of water a homologous series of methyl ketones from C_3 to C_{15} is produced (Wong, Patton & Forss, 1958; Patton & Tharp, 1959). Exhaustive distillation indicated that only limited quantities of the methyl ketones could be obtained (about 0·88 μmole/g. of milk fat) and that the precursors were thus present in relatively small amounts (Lawrence, 1963). Further, it has been shown that the C_5–C_{15} series of methyl ketones originate from the even-numbered β-oxo acids with 6–16 carbon atoms (Van der Ven, Begemann & Schogt, 1963), which are present as triglyceride components in milk fat containing one β-oxo acid and two fatty acid moieties (Parks, Keeney, Katz & Schwartz, 1964).

Preliminary work (Lawrence & Hawke, 1963) indicated that these triglyceride-bound β-oxo acids arise as intermediates in the biosynthesis of fatty acids from acetate, since the homologous series of methyl ketones obtained from the milk fat of a cow that had been given an intravenous injection of sodium [1-^{14}C]acetate possessed a labelling pattern similar to that obtained for fatty acids of similar chain length from goat milk fat (Popják, French, Hunter & Martin, 1951).

When *n*-[1-^{14}C]valeric acid was injected intravenously into a lactating cow two maxima occurred in the distribution of ^{14}C-label in the fatty acids of the milk fat (Gerson, Hawke, Shorland & Melhuish, 1960). From this it was concluded that the volatile fatty acids up to C_{10} were synthesized predominantly in the udder and that the C_{12}–C_{18} fatty acids were synthesized predominantly elsewhere. These results contrasted with those obtained by Popják *et al.* (1951), in which the specific activities of the fatty acids showed only one maximum at any given time. In view of these results, T. Gerson & F. B. Shorland commenced a further experiment in which 5 mc of [1-^{14}C]acetate was injected into the jugular vein of a cow and the specific activities of the fatty acids in the milk fat were determined for comparison with the above-mentioned experiments. Milk fat from this work was made available to the present authors in order to further their work on the origin of the methyl ketones obtained on distillation. In addition, T. Gerson & F. B. Shorland, pending publication of their results, have made available to us data relating to the specific activities of the fatty acids. This has enabled the labelling pattern of the methyl ketones to be compared directly with the labelling pattern of the fatty acids from the same source and to determine whether the methyl ketone precursors are being formed during synthesis or oxidation of fatty acids.

MATERIALS AND METHODS

A lactating Jersey dairy cow, after milking, was injected in the jugular vein with 5·0mc of [1-^{14}C]acetate. The cow was milked 3, 9 and 22hr. after injection, the milk yields and activities of the milk fats being shown in Table 1. The milk fats were stored at −10° until required for analysis.

Chemicals. Sodium [1-^{14}C]acetate and *n*-[^{14}C]hexadecane were obtained from The Radiochemical Centre, Amersham, Bucks., activated magnesia (Seasorb 43 and Magnesia 2665) was from Fischer Scientific Co., Silver Spring, Md., U.S.A., Celite 545 was from Johns–Manville Co., New York, N.Y., U.S.A., and 2,5-diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)benzene were from Nuclear Enterprises (G.B.) Ltd., Edinburgh.

Isolation and separation of fatty acids and measurement of their ^{14}C activities. By using the procedure described by Gerson *et al.* (1960) the fatty acids of the milk fat were separated into steam-volatile and non-volatile acids, the latter being further resolved into 'solids' and 'liquids'.

The steam-volatile acids were separated by gas–liquid chromatography, as were the solids and liquids, as their methyl esters. The specific activities of the purified fractions were determined in a liquid-scintillation counter (T. Gerson & F. B. Shorland, personal communication).

Isolation of methyl ketones. A 20g. sample of the milk fat from each of the three milkings was steam-distilled at 100°. It was necessary to collect 10l. of steam-distillate to ensure that the maximum quantities of methyl ketones were obtained. However, only the fat from the second milking was steam-distilled exhaustively because the volume of liquid in the distilling flask increases slowly as the distillation proceeds, making complete recovery of higher methyl ketones a lengthy process. Table 2 shows that, except for the lower yields of C_{15} methyl ketone, the yields of the methyl ketones did not seem to be affected by the shorter time of distillation.

The distillates were led directly into 2,4-DNP-hydrazine reagent (2g./l. of 2N-HCl) at 0°, and the distillate-reagent mixture was kept at room temperature in a stoppered container for 24hr. The 2,4-DNP-hydrazones were then extracted with carbonyl-free hexane, prepared according to the method of Schwartz & Parks (1961), and the extract was evaporated to dryness.

Purification of methyl ketones. The 2,4-DNP-hydrazones were subjected to a series of chromatographic procedures to remove neutral fat, fatty acids, alkanals and alkenals, which, as trace contaminants, might have contributed to the radioactivity of the 2,4-DNP-hydrazones of the methyl ketones.

Traces of neutral fat were removed from the 2,4-DNP-hydrazones by using a column of a 1:1 mixture of activated magnesia (Seasorb 43) and Celite 545. Neutral fat was eluted in hexane (Schwartz, Haller & Keeney, 1963), and the 2,4-DNP-hydrazones were eluted with nitromethane–chloroform (1:3, v/v). The 2,4-DNP-hydrazones were then added to an alumina column (2g. packed in about 4ml. of chloroform) in a small volume of chloroform and the column was eluted with further chloroform until the effluent became colourless. Any contaminant fatty acids remained on the column.

To separate the 2,4-DNP-hydrazones of the methyl ketones from the hydrazones of other carbonyls the dry effluent from the alumina column was taken up in hexane–chloroform (17:3, v/v) and added to a column containing a 1:1 mixture of Magnesia 2665 and Celite 545, according to the method of Schwartz, Parks & Keeney (1962). The 2,4-DNP-hydrazones of the methyl ketones were eluted with the same solvent mixture.

Naturally occurring alkanals and alkenals have been reported in milk fat (Parks, Keeney & Schwartz, 1961) and were isolated in steam-distillates. In the present investigation small amounts of alka-2,4-dienals were also detected, indicating chemical deterioration during storage at −10°. The concentration of carbonyls other than methyl ketones varied between 5 and 10% of the total carbonyls obtained, but were not radioactive. Methyl ketones have not, however, been reported as products of the autoxidation of fats.

Separation of methyl ketones. The isolated 2,4-DNP-hydrazones of the methyl ketones were evaporated to dryness, taken up in a minimum amount of hexane and separated into individual compounds on Celite columns by eluting with nitromethane–hexane mixtures (Day, Bassette & Keeney, 1960). The fractions were identified and their purity was established by the paper-chromatographic methods of Klein & de Jong (1956) and Huelin (1952). The concentrations of methyl ketones were determined spectrophotometrically at 345mμ as the 2,4-DNP-hydrazones in hexane.

Determination of specific radioactivities. Isolated lipid fractions and the 2,4-DNP-hydrazones (less than 0·2mg.) were counted in a liquid-scintillation counter (assembly NE8301; Nuclear Enterprises Ltd.) in toluene solution containing 0·05% of 2,5-diphenyloxazole and 0·03% of 1,4-bis-(5-phenyloxazol-2-yl)benzene.

Quenching corrections for 2,4-DNP-hydrazones were established by using *n*-[^{14}C]hexadecane as an internal standard. Quenching curves were prepared for the 2,4-DNP-hydrazones of the C_3–C_{15} methyl ketones. In all cases the quenching of radioactivity was found to be proportional to the amount of 2,4-DNP-hydrazone added, up to a maximum of 0·2mg. The corrections necessary to compensate for quenching were calculated from the quenching curves and, also as a check, by adding a known amount of the standard *n*-[^{14}C]hexadecane dissolved in toluene to the vial containing the radioactive 2,4-DNP-hydrazone.

Correction factors necessary to compensate for quenching by the steam-volatile fatty acids and methyl esters were never greater than 1·1.

Table 1. *Milk yield and milk-fat radioactivity after the injection of* [1-^{14}C]*acetate*

Milk sample	Time after injection (hr.)	Milk yield (oz.)	Sp. activity of milk fat (μc/g.atom of C)
1	3	24	2·27
2	9	88	12·00
3	22	88	4·92

RESULTS

Table 2 shows the yields of methyl ketones obtained by steam-distillation of fat from each of the three milkings. No methyl ketones with carbon

Table 2. *Yields of methyl ketones obtained by steam-distillation at 100° of milk fat from a cow milked 3, 9 and 22 hr. after the intravenous injection of 5 mc of [1-^{14}C]acetate*

A 6l. volume of distillate was collected from each of the 3hr. and 22hr. milkings, and 10l. was collected from the 9hr. milking.

Methyl ketone	Time of milking after injection.....	Yield of methyl ketone (μmole/g. of milk fat) 3hr.	9hr.	22hr.
C_3		0·31	0·34	0·25
C_5		0·07	0·10	0·11
C_7		0·12	0·12	0·06
C_9		0·08	0·07	0·05
C_{11}		0·06	0·08	0·08
C_{13}		0·09	0·09	0·09
C_{15}		0·09	0·17	0·09
Total C_5–C_{15}		0·51	0·63	0·48

number greater than C_{15} were detected in any of the distillates.

Fig. 1 shows the relationship between the specific activities of the individual methyl ketones obtained from milk fat from the three milkings and the chain length of the methyl ketones. The very similar distribution of ^{14}C between the methyl ketones in the second and the third milkings contrast with that of the first milking. Whereas in the first milking the relatively low specific activities of the methyl ketones decrease from 2·65 μc/g.atom of C to 0·72 μc/g.atom of C, the higher specific activities of the methyl ketones in the other two milkings increased to a maximum at C_{11} (22·5 and 12·5 μc/g. atom of C for the second and third milkings respectively). The specific activities of tridecan-2-one and pentadecan-2-one in the third milking were considerably greater than for the ketones below C_{11}.

Although appreciable proportions of acetone were obtained in the steam-distillates of each milking (Table 2), radioactivity was detected only in the acetone from the second milking and even in this sample the acetone had a very low specific activity.

The fatty acid composition of the milk fat, as shown by relative peak areas on gas–liquid chromatograms, fell within the range normally found (e.g. Hawke, 1957). In addition, propionic acid and a C_5 acid, assumed to be valeric acid, were also detected in trace amounts but their radioactivities were not determined.

Fig. 1 also shows the distribution of ^{14}C in the fatty acids of milk fat obtained from the three milkings. The fatty acids from the fat of the first milking (3hr. after injection) had uniformly low specific activities, the C_6–C_{12} acids having the highest activity (about 0·85 μc/g.atom of C). The

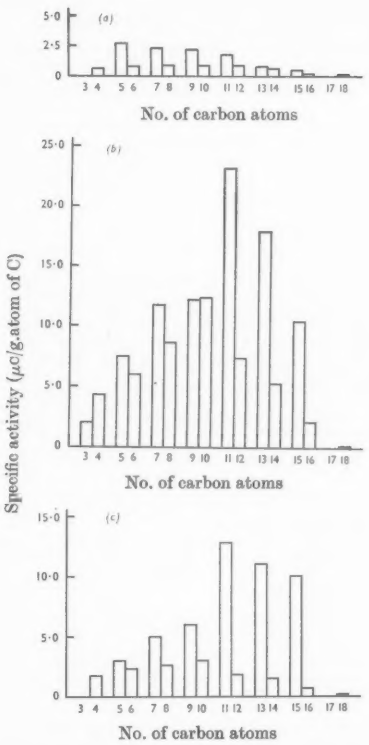


Fig. 1. Relationship between the specific activity and the number of carbon atoms in the fatty acids (even number of carbon atoms) and the methyl ketones (odd number of carbon atoms) isolated from the milk fat of a lactating cow injected with [1-^{14}C]acetate 3hr. (*a*), 9hr. (*b*) and 22hr. (*c*) before milking.

fatty acids of highest radioactivity were in the second milking (9hr. after injection), peak activity being found at C_{10} (12·9 μc/g.atom of C).

The pattern of labelling of the fatty acids of the third milking, 22hr. after injection, was almost identical with that of the second milking, with peak activity again at C_{10} acid (2·94 μc/g.atom of C). The specific activities were, however, three- to four-fold lower than in the second milking, whereas there was only a two- to three-fold decrease in specific activities of the methyl ketones from the second to the third milking. Except for acetone in all milkings and C_9 methyl ketone in the second milking, the methyl ketones had greater specific activities than the corresponding fatty acids. In the two later milkings comparisons of labelling in

fatty acids and methyl ketones show that the principal features were the close similarities in the specific activities of the two series of compounds in the C_5–C_{10} range and the much higher specific activities of the methyl ketones in the C_{11}—C_{16} range.

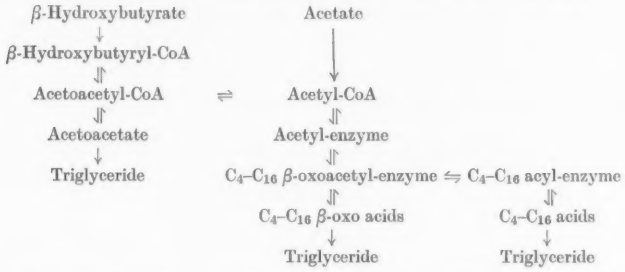
DISCUSSION

Although there are several points of difference in the labelling patterns of the fatty acids and the corresponding methyl ketones with one less carbon atom, the results in general are consistent with the view that the β-oxo acids, from which the methyl ketones are formed during steam-distillation, form part of the same metabolic pool as the fatty acids undergoing incorporation into triglycerides. Consequently, the higher methyl ketones are very weakly labelled in the first 3hr., yet contain the major part of the activity at the end of 22hr. The change in labelling pattern of the fatty acids with time is in accord with similar experiments with the goat by Popják *et al.* (1951), and points to the existence of a relatively large pool of fatty acids in the mammary gland and to the relatively slow rate of secretion of fat and fat precursors in the alveoli after its synthesis in the cells (Hardwick, Linzell & Mepham, 1963).

The high specific activities of the methyl ketones relative to the corresponding fatty acids may be a reflection of the formation of β-oxo acids as intermediates in fatty acid synthesis prior to a rate-limiting step or of a greater rate of incorporation of the β-oxo acids than of the fatty acids into the triglycerides. The close similarity between the labelling patterns and the specific activities of the low-molecular-weight fatty acids and the corresponding methyl ketones with one less carbon atom in the last two milkings suggest that the β-oxo acids and the corresponding fatty acids have a common precursor and are together incorporated into the triglycerides. Insufficient evidence is available to give a reliable explanation of the finding that the specific activities of the C_{12}–C_{16} fatty acids in the 9hr. and 22hr. milkings are considerably lower than the activities of the corresponding methyl ketones. A possible explanation is that there is a dilution of the labelled fatty acids being synthesized from acetate in the mammary gland by fatty acids synthesized by less direct pathways, or that the differences are related to the size of the metabolic pools. The higher specific activities of the methyl ketones relative to the fatty acids is evidence that the β-oxo acids are not synthesized from fatty acids by β-oxidation. The relationship between the labelling of fatty acids or methyl ketones when radioactive acetate is used provides additional understanding of the biosynthetic origin of the fatty acid components in milk. The generally accepted view (e.g. Folley & McNaught, 1961) is that in the biosynthesis of milk the fatty acids, up to and including palmitic acid, are synthesized from an acetate pool, whereas C_{18} acids and above are synthesized by less direct pathways or from the blood triglycerides (Dils & Popják, 1962). It appears that, apart from acetoacetate, the β-oxo acids which are incorporated into milk triglycerides arise from the direct utilization of acetate in the mammary gland, because neither radioactive nor unlabelled C_{17} methyl ketone or higher-molecular-weight ketones were detected in the milk fat, and stearic acid had extremely low specific activities in all milkings. The presence of small amounts of β-oxo acid in triglycerides with similar specific activity to fatty acids of the same carbon number implies that there is some dissociation of the β-oxoacyl-enzyme (Lynen, 1961) or β-oxoacyl-*S*-acyl-carrier protein (Alberts, Majerus, Talamo & Vagelos, 1964) in the mammary gland before the dissociation of acyl-enzyme or acyl-(acyl-carrier protein) during triglyceride synthesis. In an extension of the present study coconut oil, which like milk fat is characterized by a high proportion of saturated C_8–C_{16} acids, was also steam-distilled, but no methyl ketones exept acetone were found. This suggests that any C_6–C_{16} β-oxo acids incorporated into the triglycerides of coconut oil are presumably reduced *in situ* and that the presence of β-oxo acids in the triglycerides of milk fat possibly results from their rapid removal from the site of synthesis during milk secretion.

Though most attention has been given to the reduction of D-β-hydroxybutyric acid of the blood stream to butyric acid (e.g. Popják *et al.* 1951; Kumar, Lakshmanan & Shaw, 1959; Annison, Leng, Lindsay & White, 1963), the results of Black, Kleiber & Brown (1961) indicate that the cow can metabolize butyrate via the classical β-oxidation pathway and the tricarboxylic acid cycle. Similarly, the present work suggests that the mammary gland can oxidize β-hydroxybutyric acid to acetoacetic acid, because in all milkings butyric acid had a higher specific activity than the corresponding methyl ketone. The presence of unlabelled acetoacetate, but labelled higher β-oxo acids, in milk triglycerides after the injection of labelled acetate could be explained by the pathway outlined in Scheme 1 and based on the mechanism for fatty acid synthesis proposed by Bressler & Wakil (1962). The quantitative importance of acetone in the mixed methyl ketones from milk fat suggests that more acetoacetate than any of the higher β-oxo acids is present in the metabolic pool.

Since both the C_5–C_{15} methyl ketone precursors and the short-chain fatty acids in milk fat are synthesized from acetate, the amounts of methyl ketone obtained on distillation might be expected to exhibit a seasonal variation similar to that



Scheme 1. Hypothetical mechanism for the formation and incorporation of β-oxo acids into milk fat.

observed with the quantitatively important short-chain fatty acids (Hansen & Shorland, 1952; Keeney, 1956). The greater amounts of C_5–C_{15} methyl ketones reported by Lawrence (1963) in samples of New Zealand butterfat (about 0·88 μmole/g. compared with 0·63 μmole/g. in the present study) might have been due to a seasonal effect.

The very low radioactivity of the unsaturated C_{18} fatty acids appears to exclude the possibility, suggested by Wong *et al.* (1958), that methyl ketones are products of the autoxidation of milk fat.

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Observations on the Biosynthesis of Thiamine in Yeast

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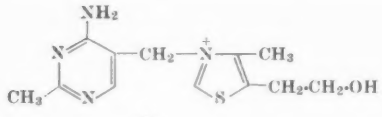
(*Received* 27 *April* 1965)

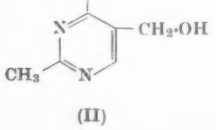
1. Methods are described for the isolation of radioactively pure thiamine from yeast and its degradation on a small scale to its cyclic components. 2. A degradation of the pyrimidine ring and a thin-layer method for the separation of thiamine, its derivatives and pyrimidine and thiazole residues are described. 3. [^{14}C]Formate is more effectively incorporated into the pyrimidine residue than into the thiazole residue, whereas the reverse is true with L-[*Me*-^{14}C]methionine. 4. Experiments with [*Me*-^{14}C,^{35}S]methionine demonstrate that methionine provides an intact unit for the biosynthesis of the thiazole ring. 5. [6-^{14}C]Orotic acid is insignificantly incorporated into the pyrimidine residue of thiamine. 6. Experiments with [1-^{14}C]- and [2-^{14}C]-acetate indicate that it is incorporated as a unit into the thiazole residue, but that only C-2 is incorporated into the pyrimidine residue. 7. L-[U-^{14}C]Alanine is also effectively incorporated into the thiazole residue. 8. These results are discussed in relation to possible pathways of biosynthesis of the two ring components of the thiamine molecule.

Early nutrition experiments of Schopfer & Jung (1937) and Robbins & Kavanagh (1937) showed that the mould *Phycomyces blakesleeanus*, which required thiamine (I) for growth, would also grow on a mixture of the pyrimidine (4-amino-5-hydroxymethyl-2-methylpyrimidine) (II) and the thiazole [5-(2-hydroxyethyl)-4-methylthiazole] (III) residues of thiamine. Later it was found that thiamine-requiring fungi fell into four distinct categories according to whether they required: (1) preformed thiamine; (2) both compound (II) and compound (III) preformed; (3) only compound (II) preformed; (4) only compound (III) preformed (for full details see Schopfer, 1943; Goodwin, 1963). These investigations clearly indicated that the final stages of thiamine biosynthesis involved condensation of the two preformed ring systems, and recently this has been demonstrated enzymically; the reactions involve the pyrophosphorylation of compound (II), the phosphorylation of compound (III) and the condensation of these two compounds with the liberation of pyrophosphate to form thiamine monophosphate, which is converted into thiamine by a specific phosphatase (Leder, 1959; Camiener & Brown, 1959, 1960; Nose, Ueda & Kawasaki, 1959; Suzuoki & Kobata, 1960; Lewin & Brown, 1961). Much less was known of the origin of the ring systems themselves and nutrition experiments were inconclusive (see Goodwin, 1962). However, Pine & Guthrie (1959) found that *Bacillus subtilis* A.T.C.C. 6051 incorporates [^{14}C]formate specifically into the pyrimidine fragment of thiamine; somewhat similar results were reported for baker's yeast by David & Estramareix (1960, 1961), who also stated that the label was not incorporated into position 5 of the pyrimidine residue, and for *Escherichia coli* (Goldstein & Brown, 1963). Pine & Guthrie (1959) and David & Estramareix (1960, 1961) also found [^{14}C]formate to be insignificantly incorporated into the thiazole residue of thiamine in *B. subtilis* and baker's yeast.

The present investigations were undertaken in an attempt to throw more light on the origin of the two ring components in thiamine. A preliminary



(I)



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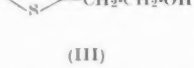


(III)

short report of part of this work has already been published (Johnson, Howells & Goodwin, 1964).

EXPERIMENTAL

Organism. Saccharomyces cerevisiae Bunker strain no. 39916 was used throughout the investigation. It was maintained on Saboraud agar slopes that, after incubation at 28±1° for 2 days, were stored at 4° until required. The liquid culture medium consisted of (per 100ml.): glucose, 2·0g.; $(NH_4)_2SO_4$, 0·0943g.; KH_2PO_4, 0·05g.; $MgSO_4,7H_2O$, 0·05g.; $CaCl_2,2H_2O$, 0·03g.; yeast extract, 0·2g. It was dispensed (150ml.) into Roux bottles that, after inoculation with a mother culture, were incubated for 24hr. at 28±1°. Radioactive material was added at this point and the incubation was continued for a further required period of time before the cells were harvested in a Sharples Super centrifuge with a roll of cellophan paper inside the collecting cylinder. The resulting thick suspension was transferred to a weighed centrifuge tube and centrifuged in a bench centrifuge (Martin Christ) at 3500 rev./min. The pellet was washed thoroughly with water, frozen, transferred to a vacuum desiccator and freeze-dried over conc. H_2SO_4. Yields were 2·6–3·0g. of dry cells/l. of medium and the thiamine content was 160–200μg./g. of dry cells.

Extraction, purification and assay of thiamine. A known weight (about 3g.) of dry cells was extracted with 75ml. of 0·1N-HCl on a boiling-water bath for 30min. The extract was cooled and clarified by centrifugation. The extraction vessel was washed with dilute HCl, the extract and washings were combined and the pH was adjusted to pH4·5. The thiamine monophosphate present in the extract was hydrolysed with Taka-diastase (Parke, Davis and Co. Ltd., Hounslow, Middlesex). Taka-diastase preparations were made by suspending 6g. of dry powder in 100ml. of 2·5M-sodium acetate; 5ml. of the fresh suspension was added to the HCl extract (75ml.) and the pH adjusted, if necessary, to 4·5. The mixture was incubated in an enzyme bath for 2·5hr. at 45–50° with intermittent shaking and then clarified by centrifugation (Martin Christ bench centrifuge). The supernatant was removed and the residue washed with dilute aqueous HCl (pH4·5). The combined supernatant and washings were then purified on columns of suitably activated Decalso F resin. The activated resin was prepared by first soaking 100g. in hot 3% (v/v) acetic acid (250ml.) for 15min.; the acid was drawn off under mild suction and the washing process repeated. The resin then was washed with hot 25% (w/v) KCl (250ml.), filtered off and the acid wash again repeated. Finally, the resin was washed with hot distilled water (600ml. portions) until free from Cl^- ions, dried at room temperature and stored in a glass jar.

Columns were made of glass tubes (inner bore 7mm. and 30cm. long) drawn out at one end to form constricted delivery jets. The narrow ends were plugged with non-absorbent cotton wool, and activated Decalso F (6g.) was poured into the tubes. The delivery jets were closed with rubber tubing and screw clips, and the tubes were filled with deionized water. The open end of each tube was closed with a clean cork, and the tubes were then shaken and inverted to free the resin bed from air bubbles. The bubble-free resin was allowed to settle and plugs of non-absorbent cotton wool were placed on the top of each resin bed, which was charged with thiamine solution and connected to dropping funnels by means of polythene tubing.

The efficiency of each batch of resin was checked by passing standard solutions (1·0–2μg./ml.) of thiamine at pH4·5 through two columns of the resin; the columns were washed with hot deionized water (30ml.) and the thiamine was eluted from each column with 25% (w/v) KCl in 0·1N-HCl (50ml.). The columns were considered satisfactory if a recovery off the second column of 94–98% was obtained.

The extracts from the Taka-diastase incubations were purified by passing 25ml. of extract (25–50μg. of thiamine) through two columns as just described. Two columns are necessary to be certain of removing all impurities that might interfere with the quantitative estimation of thiamine in an EEL fluorimeter (Association of Vitamin Chemists, 1947).

The extracts prepared as described above required further purification for radioisotope work. The eluates were desalted by a method involving extraction from the aqueous phase into phenol and re-extraction into pure water (Iacono & Johnson, 1957; Carlson & Brown, 1961). The desalted solution was evaporated to dryness in a rotary evaporator (Wrights Scientific Ltd., London), and the residue was dissolved in ethanolic HCl (pH4·0) and made up to volume (10 or 25ml.). It was at this stage that carrier thiamine was added if required.

Disulphite cleavage of thiamine. This reaction was described by R. J. Williams in his classical work on the structure of thiamine (see Williams, Eakin, Beerstecher & Shive, 1950). In the present investigation it was carried out by dissolving thiamine (100mg.) in 2·6M-Na_2SO_3 (1·5ml.) [prepared by dissolving 32·8g. of $Na_2SO_3,7H_2O$ (analytical grade) in 100ml. of deionized water and adjusting the pH to 4·8–5·0 by bubbling SO_2 through the solution] and allowing the solution to stand in a sealed centrifuge tube for 4–5 days. The sulphonate of the pyrimidine residue ($PySO_3H$) crystallized out during that time and was collected by centrifugation. The precipitate was transferred to a second centrifuge tube, washed with small volumes of cold water and recrystallized in hot water.

The washings were added to the supernatant and the pH was adjusted to 10 with 15% (w/v) NaOH. The solution was then extracted seven times with $CHCl_3$ (analytical grade) (50ml.) and the combined extracts were re-extracted with 1·5N-HCl. This solution was evaporated to dryness *in vacuo* and the residue extracted with ethanol; the extract was evaporated to dryness *in vacuo*, when the hydrochloride of the thiazole residue of thiamine (ThHCl) crystallized out. It was recrystallized by dissolving it in a minimum volume of ethanol, adding excess of dioxan (analytical grade) and allowing to stand.

Chromatography of thiamine and its derivatives. A thin-layer system was devised that will effectively separate thiamine, $PySO_3H$ and ThHCl, as well as α-hydroxythiamine and thiochrome. The adsorbent was Kieselgel G (E. Merck A.-G., Darmstadt, Germany) and the solvent pyridine–2-methylpropan-1-ol (4:1:1, by vol.). Separation was complete within 2hr. The R_F values normally obtained were: thiamine, 0·03; α-hydroxythiamine, 0·22; $PySO_3H$, 0·70; ThHCl, 0·30; thiochrome, 0·98. For the separation of ThHCl and $PySO_3H$ only the solvent system pyridine–water (4:1, v/v) was very effective (R_F 0·38 and 0·65 respectively) and was used in the experiments on the disulphite cleavage of thiamine. Separation of thiamine and its two

derivatives could also be achieved by paper chromatography on Whatman no. 1 paper with water-saturated butan-1-ol containing 18% (w/v) of formic acid. The R_F values were: thiamine, 0·019; $PySO_3H$, 0·066; ThHCl, 0·221. If thiamine and $PySO_3H$ were not clearly separated in this system the combined spots were eluted and run in pyridine–2-methylpropan-1-ol–water (2:3:5, by vol.), when the R_F values were: thiamine, 0·51; $PySO_3H$, 0·62. The advantages of the thin-layer systems over the paper systems are: (*a*) speed, the running time being 2 hr. compared with 24 hr.; (*b*) the greater sensitivity in radioautography, activities that will show up after overnight exposure on thin layers requiring some 2–3 weeks' exposure when adsorbed on paper (see Goodwin, 1964).

The compounds could be seen on the chromatograms under u.v. light (Hanovia lamp, model 16, with Chance O × 1 filter). $PySO_3H$, ThHCl and thiochrome spots exhibited light-blue, dark-mauve and bright-blue fluorescence respectively. Thiamine and α-hydroxythiamine show up as dark absorbing spots; they are more easily detectable if they are first converted into their thiochrome derivatives by spraying the chromatogram with a 5:5:1 (by vol.) mixture of aq. 55% (v/v) ethanol, 10% (w/v) NaOH and 2·5% (w/v) $K_3Fe(CN)_6$ (Rossi-Fanelli, Siliprandi & Fasella, 1952).

The eluted $PySO_3H$ and ThHCl were eluted with 0·1 N-NaOH and ethanol respectively, and assayed spectrophotometrically in 0·1 N-NaOH and 0·5 N-HCl respectively: $PySO_3H$, $\lambda_{max.}$ 235·5 ($E^{1\%}_{1cm.}$ 220) and 277·5 mμ ($E^{1\%}_{1cm.}$ 425); ThHCl, $\lambda_{max.}$ 260 mμ ($E^{1\%}_{1cm.}$ 190).

In experiments with radioactive thiamine the purity of the two ring components was always checked by making a radioautograph of a thin-layer chromatogram.

Degradation of $PySO_3H$. The method of Lagerkvist (1953) for the degradation of uracil was adapted for use with $PySO_3H$. The $PySO_3H$ (0·1–0·15 m-mole) was dissolved in 3 ml. of 0·01 N-NaOH, and 5% (w/v) $KMnO_4$ was added dropwise until a faint permanganate colour persisted. The solution was acidified with 0·6 ml. of 0·1 N-H_2SO_4 and allowed to stand for 30 min. Excess of $KMnO_4$ was destroyed with H_2O_2, and the precipitated MnO_2 was removed by centrifugation. The precipitate was washed with small volumes of deionized water, and the combined supernatant and washings were hydrolysed by adding 1 ml. of 5 N-NaOH and refluxing on a water bath for 3 hr. The solution was cooled, acidified with 5 N-H_2SO_4 and steam-distilled. The steam-volatile fraction was examined by gas–liquid chromatography (see the next section).

Gas–liquid chromatography. The volatile acids from the degradation of $PySO_3H$ were separated in an Argon Gas Chromatograph (Pye Instruments Ltd., Cambridge) with a 4 ft. column of 12% polyethylene glycol adipate in 80–100 mesh Gas-Chrom P (silicone-treated). The detector voltage was 1250 v, sensitivity × 10, temp. 110° and gas flow rate 50 ml./min. Known volumes of material in ether were transferred to pads of flamed gauze from a micrometer syringe (Agla; Burroughs, Wellcome and Co., London), and the pads were dropped into place on the column top.

Radiochemical analysis. Radioactive materials were assayed in either (*a*) a Nuclear–Chicago Corp. automatic sample changer (model C 110B) used in conjunction with a decade scaler (model 186; Nuclear–Chicago Corp.), the counting chamber being fitted with a Micromil window and flushed with proportional counting gas (argon–methane, 9:1), and the efficiency of the system being 17·5%, or (*b*) a Packard Tri-Carb liquid-scintillation spectrometer (model 314 Ex).

Radioautography of thin layers was carried out conventionally with standard X-ray film.

The effluent from the gas chromatogram was scanned for radioactivity in a gas proportional counter (Pye Instruments Ltd.) coupled to a recording rate-meter (type N 701A; Honeywell–Brown Electronik).

RESULTS

Incorporation of formate. The results of an experiment in which labelled formate was added to growing yeast cells are given in Table 1. This experiment was designed to give sufficient thiamine of high radioactivity that could be isolated without the addition of unlabelled carrier. This was to allow the degradation of the isolated pyrimidine-sulphonic acid and the assay of the volatile products for radioactivity. Table 1 clearly indicates that formate is significantly incorporated only into the pyrimidine residue of thiamine; the specific activity of the pyrimidine residue is 100 times that of the thiazole residue. Oxidative degradation of the $PySO_3H$ yielded acetic acid and, probably, propionic acid as the two volatile products when examined in a gas chromatogram coupled with a radioactive scanner; essentially all the radioactivity was associated with the acetic acid peak. When the machine was overloaded with radioactivity so that the peak of activity due to acetic acid was well off the scale, a trace of activity was noted in the second zone. From this experiment it can be concluded that the methyl group attached to C-2 of the pyrimidine residue arises directly from

Table 1. *Incorporation of* [^{14}C]*formate into thiamine and its two ring components by baker's yeast*

A 4·0 mc sample of [^{14}C]formate (specific activity 13·4 mc/m-mole) was added to 4·5 l. (in 30 Roux bottles) of yeast cells 24 hr. after inoculation; the cells were incubated for a further 96 hr. at 28°.

Yield of dry cells	18·2 g.
Yield of thiamine	3·82 mg.
Activity of thiamine	$8·8 \times 10^7$ disintegrations/min.
Specific activity of thiamine	$7·93 \times 10^6$ disintegrations/min./μmole
Yield of $PySO_3H$	1·54 mg.
Activity of $PySO_3H$	$5·02 \times 10^7$ disintegrations/min.
Specific activity of $PySO_3H$	$7·85 \times 10^6$ disintegrations/min./μmole
Yield of ThHCl	0·95 mg.
Specific activity of ThHCl	$0·08 \times 10^6$ disintegrations/min./μmole

Table 2. *Incorporation of* L-[Me-^{14}C]*methionine into thiamine by baker's yeast*

A 0·1 mc sample of L-[*Me*-^{14}C]methionine (specific activity 10·9 mc/m-mole) was added to 1·5 l. (in ten Roux bottles) of yeast cells 24 hr. after inoculation; the cells were incubated for a further 12 hr. at 28°.

Yield of dry cells	2·59 g.
Yield of thiamine	0·619 mg.
Specific activity of thiamine	$3·78 \times 10^4$ disintegrations/min./μmole
Specific activity of $PySO_3H$	$0·96 \times 10^3$ disintegrations/min./μmole
Specific activity of ThHCl	$1·93 \times 10^3$ disintegrations/min./μmole
Specific activity ratio $PySO_3H$/ThHCl	0·53:1

formate, that none of the carbon atoms of the thiazole ring arises directly from formate and that the C_1 fragment attached to C-5 may or may not be derived from formate. This last conclusion remains to be settled, because it is still not clear from where in the molecule the unlabelled propionic (?) acid arises.

Incorporation of methionine into thiamine. Table 2 shows the results of an experiment in which it is demonstrated that [*Me*-^{14}C]methionine is more effectively incorporated into the thiazole residue of thiamine than into the pyrimidine residue. This strongly suggested that methionine was not functioning as a source of a C_1 unit in the thiazole residue but providing a much larger building unit. This view was supported when it was found (Table 3) that [^{35}S]methionine was better incorporated than was [^{35}S]cysteine, in spite of the fact the cysteine was more efficiently taken into the yeast cells. To make conditions as comparable as possible cells from the same batch were used under identical conditions for this experiment. Further, this precluded the need to allow for the decay of ^{35}S.

These results prompted an examination of the incorporation of L-[*Me*-^{14}C,^{35}S]methionine into the thiazole residue of thiamine. As the ^{35}S/^{14}C ratio in the added methionine was known, then, if the total counts in the isolated thiazole are measured over a considerable time (preferably longer than the half-life of ^{35}S, namely 87 days) and the logarithm of the counts plotted against time, and the activities at zero time and at 87 days determined from the graph, the ^{35}S/^{14}C ratio in the thiazole at zero time can be determined and compared with that of the original methionine. If they are the same then methionine must be providing an intact unit for thiazole biosynthesis and not just a C_1 residue.

An experiment was carried out under our standard conditions (Table 4) in which the ^{14}C/^{35}S ratio in the methionine added to the culture was 1:1. Samples of the thiazole residue obtained from the thiamine synthesized in this experiment were plated out on three sets of duplicate planchets. The same amount of material was added to each planchet in the same set, but the amounts added to each set were different. Thus the same basic sample was counted at three different levels of activity. On every occasion each planchet was counted ten times on a preset count system arranged so that the counting time was approx. 10 min.; the period covered was 110 days. The results for the three series are given in Table 5 and the plot for series I is recorded in Fig. 1. From this graph $\log D_0 = 4·0025$ and $\log D_{87} = 3·8812$, so that $D_0 = 10060$ and $D_{87} = 7610$. The difference, which represents one-half the original activity of ^{35}S, is 2450; the activity of

Table 3. *Comparison of incorporation of* DL-[^{35}S]*methionine and* DL-[^{35}S]*cystine into thiamine by baker's yeast*

A 0·13 mc sample of DL-[^{35}S]methionine (specific activity 15 mc/m-mole) or a 0·15 mc sample of DL-[^{35}S]cystine (specific activity 18 mc/m-mole) was added to 1·5 l. (in ten Roux bottles) of yeast cells 24 hr. after inoculation; the cells were incubated for a further 12 hr. at 28°.

	With DL-[^{35}S]methionine	With DL-[^{35}S]cystine
Yield of dry cells	2·99 g.	3·02 g.
Yield of thiamine	0·207 mg.	0·218 mg.
Specific activity of thiamine	$5·6 \times 10^4$ disintegrations/min./μmole	$1·32 \times 10^3$ disintegrations/min./μmole
Percentage of added activity left in medium	61	36·5

Table 4. *Incorporation of* L-[Me-^{14}C,^{35}S]*methionine into thiamine by baker's yeast*

A 1 mc sample of L-[*Me*-^{14}C]methionine (specific activity 6·3 mc/m-mole) and a 1 mc sample of L-[^{35}S]methionine (specific activity 9·3 mc/m-mole) were added to 4·5 l. (in 30 Roux bottles) of yeast cells 24 hr. after inoculation; the cells were incubated for a further 48 hr. at 28°.

Yield of dry cells	6·5 g.
Yield of thiamine	1·230 mg.
Activity of thiamine	$1·53 \times 10^8$ disintegrations/min.
Specific activity of thiamine	$4·25 \times 10^7$ disintegrations/min./μmole
Yield of ThHCl	0·365 mg.
Activity of ThHCl	$3·54 \times 10^7$ disintegrations/min./μmole
Yield of $PySO_3H$	0·520 mg.
Specific activity of $PySO_3H$	$0·71 \times 10^7$ disintegrations/min./μmole

Table 5. *Decay of radioactivity of thiazole samples isolated from thiamine synthesized by baker's yeast in the presence of* L-[Me-^{14}C,^{35}S]*methionine*

For details of the experiment see Table 4. Three series of two planchets were assayed at the times indicated.

Days after addition of labelled methionine	Radioactivity (disintegrations/min.)		
	Series I	Series II	Series III
29	9175	16410	15400
30	9130	16220	15370
31	9120	16130	15310
35	8960	15940	15120
48	8590	15230	14510
49	8561	15140	14400
52	8511	15050	14290
55	8417	14880	14180
57	8366	14790	14050
59	8270	14710	13960
63	8220	14450	13850
65	8175	14380	13680
106	7160	12520	11950
107	7120	12470	11930
108	7110	12390	11890
109	7095	12370	11840

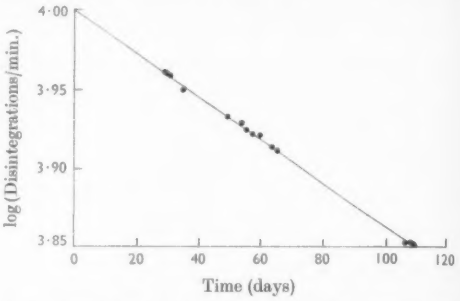


Fig. 1. Rate of decay of thiazole sample I (Table 5) isolated from thiamine synthesized by baker's yeast in the presence of L-[*Me*-^{14}C,^{35}S]methionine (^{14}C/^{35}S ratio 1:1 at time zero). For details of the experiment see Table 4.

^{35}S at time zero is thus 4900, and that of ^{14}C is 10060 − 4900 = 5160; so the ^{14}C/^{35}S ratio is 1·05:1. The ratios calculated in the same way for series II and III were 0·93:1 and 1·00:1 respectively. From this it can be concluded that methionine is providing a building unit for the biosynthesis of the thiazole ring.

Table 4 shows that some activity from [^{14}C]-methionine is incorporated into the pyrimidine residue, and this confirms the results given in Table 2 and demonstrates that to carry out the doubly labelled experiment just described it is necessary to work on the isolated thiazole residue and not on the intact thiamine molecule.

Incorporation of [6-^{14}C]*orotic acid into thiamine.* If the pyrimidine residue of thiamine arises by the conventional pathway of pyrimidine synthesis then labelled orotic acid would be expected to be actively incorporated into the pyrimidine residue. Table 6 shows that only an insignificant incorporation, 1270 disintegrations/min. from 0·2mc, was observed. This is at least 1000-fold less than the incorporation of [^{14}C]formate (see Table 1) and agrees with the observations of Goldstein & Brown (1963) with an orotic acid-dependent mutant of *E. coli*. The little activity that was observed in thiamine appeared to be confined to the pyrimidine residue.

Incorporation of acetate and alanine into thiamine. The observation that methionine was contributing a unit to the thiazole residue of thiamine suggested that N-3, C-4 and the methyl group attached to

Table 6. *Incorporation of* [6-^{14}C]*orotate into thiamine by baker's yeast*

A 0·2mc sample of [6-^{14}C]orotate (specific activity 32·6mc/m-mole) was added to 4 5l. (in 30 Roux bottles) of yeast cells 24hr. after inoculation; the cells were incubated for a further 48hr. at 28°.

Yield of thiamine	1·00mg.
Activity of thiamine	1270 disintegrations/min.
Specific activity of thiamine	
(*a*)*	428 disintegrations/min./μmole
(*b*)†	285 disintegrations/min./μmole
Yield of $PySO_3H$	0·64mg.
Activity of $PySO_3H$	760 disintegrations/min.
Specific activity of $PySO^3H$	285 disintegrations/min./μmole

* Before dilution with carrier thiamine.
† After dilution with carrier thiamine for degradation.

C-4 (see compound I) arose from a source other than methionine. Further, the failure to incorporate formate to any significant extent into the thiazole residue (Table 1) indicated that the methyl group did not arise from a C_1 unit. The possibility was therefore tested whether C-4 and its attached methyl group could arise from a C_2 unit (acetate) and whether alanine could supply these two carbon atoms and the nitrogen atom at position 3. An experiment with [1-^{14}C]acetate and [2-^{14}C]acetate demonstrated that both were incorporated into thiamine, that, under the same cultural conditions, [1-^{14}C]- and [2-^{14}C]-acetate were incorporated into the thiazole residue to an equal extent, that [1-^{14}C]-acetate was insignificantly incorporated into the pyrimidine residue, and that [2-^{14}C]acetate was

Table 7. *Incorporation of* [1-^{14}C]- *and* [2-^{14}C]-*acetate into thiamine in baker's yeast*

A 0·1 mc sample of [1-^{14}C]acetate (specific activity 12 mc/m-mole) or a 0·1 mc sample of [2-^{14}C]acetate (specific activity 10 mc/m-mole) was added to 1·5 l. (in ten Roux bottles) of yeast cells 24 hr. after inoculation; the cells were incubated for a further 48 hr. at 28°. To make conditions as comparable as possible the two batches were inoculated from the same suspension of yeast cells and incubated side by side.

	With [1-^{14}C]acetate	With [2-^{14}C]acetate
Yield of thiamine	0·336 mg.	0·477 mg.
Specific activity of thiamine after dilution	$2·64 \times 10^4$ disintegrations/min./μmole	$4·03 \times 10^4$ disintegrations/min./μmole
Yield of ThHCl	60 μg.	60 μg.
Specific activity of ThHCl	(*a*)* $2·64 \times 10^4$ disintegrations/min./μmole (*b*)† $1·69 \times 10^5$	$3·33 \times 10^4$ disintegrations/min./μmole $1·80 \times 10^5$
Yield of $PySO_3H$	140 μg.	120 μg.
Specific activity of $PySO_3H$*	0	$7·0 \times 10^3$ disintegrations/min./μmole

* Derived from undiluted thiamine.
† Calculated for undiluted material.

Table 8. *Incorporation of* L-[U-^{14}C]*alanine into thiamine by baker's yeast*

A 0·1 mc sample of L-[U-^{14}C]alanine (specific activity 15·2 mc/m-mole) was added to 500 ml. (in ten Erlenmeyer flasks) of yeast cells 24 hr. after inoculation; the cells were incubated for a further 24 hr. with shaking at 28°.

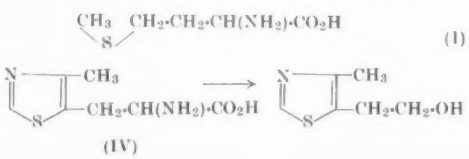
Yield of thiamine	150 μg.
Specific activity of thiamine	
(*a*)*	$1·80 \times 10^5$ disintegrations/min./μmole
(*b*)†	$2·35 \times 10^4$ disintegrations/min./μmole
Yield of ThHCl	36 μg.
Specific activity of ThHCl	
(*a*)‡	$2·28 \times 10^4$ disintegrations/min./umole
(*b*)§	$1·75 \times 10^5$ disintegrations/min./μmole
Yield of $PySO_3H$	30 μg.
Specific activity of $PySO_3H$§	$0·07 \times 10^4$ disintegrations/min./μmole

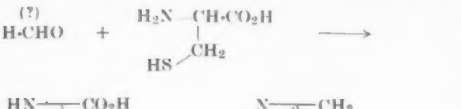
* Before dilution with carrier thiamine.
† After dilution with carrier thiamine for degradation.
‡ Derived from diluted thiamine.
§ Calculated for undiluted material.

incorporated into the pyrimidine residue but to a smaller extent than into the thiazole residue (thiazole/pyrimidine specific activity ratio 4·7:1) (Table 7). An experiment with L-[U-^{14}C]alanine (Table 8) showed clearly that the label is very effectively incorporated into the thiazole residue of thiamine, whereas only little activity was found in the pyrimidine residue, the specific activity of the thiazole residue being only about one-thirtieth that of the pyrimidine residue.

DISCUSSION

Origin of the thiazole residue. Harington & Moggridge (1940) suggested that the thiazole ring in thiamine arose from α-amino-β-(4-methylthiazol-5-yl)propionic acid (IV), which in turn could be considered formally to arise from methionine, acetaldehyde and an amino group (reaction 1); support for this view came from the observation that compound (IV) stimulated thiamine production in yeast (Harington & Moggridge, 1939) and pea roots (Bonner & Buchman, 1938); isotope competition experiments, however, indicated that compound (IV) was not an effective precursor, but nutritional studies with isolated pea roots, which require preformed thiazole, suggested that the C_2 side chain must be intact (see Bonner & Bonner, 1948). Our present experiments (Table 4) demonstrate that the CH_3–S grouping of methionine is incorporated intact into the thiazole residue, and thus it is reasonable to assume that C-5 and the hydroxyethyl side chain also come from methionine, in agreement with the proposals of Harington & Moggridge (1940). The failure of yeast to incorporate [^{14}C]formate into the thiazole residue (Table 1) indicates that the methyl group at C-4 is not derived from a C_1 unit, and the equal incorporation of [1-^{14}C]- and [2-^{14}C]-acetate into this residue (Table 7) suggests that acetate is being incorporated into the thiazole residue intact and thus is a logical precursor of C-4 and its attached methyl group; on modern biochemical views this is more likely than acetaldehyde, which was the first C_2 unit proposed. However, this does not mean that acetate is the normal precursor *in vivo*, and the effective incorporation of L-[U-^{14}C]alanine into the thiazole residue (Table 8) makes this amino acid a good candidate for the source of all the atoms not arising from methionine, although the activity may be due to its conversion into pyruvate, which, via





(V) (VI) (2)

acetate, is then incorporated. This would also require the existence of a specific amino donor, e.g. glutamate, as a source of N-3. However, the fact that the labelling pattern in the pyrimidine residue with L-[U-^{14}C]alanine is different from that with [2-^{14}C]acetate argues against the prior degradation of alanine. So it would appear that the cyclic precursor of the thiamine thiazole residue arises from combination of L-alanine and L-methionine. The nature of this precursor remains to be established but, as indicated above, nutritional studies with pea roots suggest that the C_2 side chain must be intact. On the other hand, Nakayama (1956*a*,*b*, 1957*a*,*b*) proposed reaction (2) for the biosynthesis of the thiazole residue as a result of investigations on thiamine-requiring mutants of *E. coli* and *Neurospora crassa*, which can utilize the thiazole residue. The mutants respond to a limited extent to 4-methylthiazole (VI), and can be maintained in the absence of the thiazole residue by either cysteine or thiazolidene-4-carboxylic acid (V) but not by homocysteine, methionine or methanol. Our results contrast sharply with these because we find that methionine is actively incorporated into the thiazole residue (Tables 2, 3 and 4), that [^{35}S]-methionine is more effectively incorporated than is [^{35}S]cysteine (Table 4) and that formate is ineffectively incorporated. Further, Korte & Weitkamp (1959) and Korte, Weitkamp & Vogel (1959) reported that 4-methyl[2-^{14}C]thiazole is not incorporated into thiamine in a number of microorganisms, and Hitchcock & Walker (1961) found that methionine dilutes out the incorporation of $^{35}SO_4^{2-}$ into thiamine more effectively than does cysteine.

Origin of the pyrimidine residue. A number of observations raise doubts as to whether the pyrimidine residue arises via the conventional biosynthetic pathway, i.e. through carbamoylaspartate, orotic acid and uridine monophosphate, followed by a C_1 transfer similar to that involved in thymine biosynthesis. Goldstein & Brown (1963) demonstrated that in an orotic acid-dependent *E. coli* only a very small incorporation of [^{14}C]orotic acid was observed and, further, somewhat greater incorporation was obtained with [2-^{14}C]orotic acid than with [6-^{14}C]orotic acid, which suggests that the ring is metabolized before the ^{14}C was incorporated into thiamine. Very low incorporation was also obtained by Goldstein & Brown (1963) with [U-^{14}C]uracil, [U-^{14}C]aspartic acid and [4-^{14}C]-aspartic acid. Our observations with [6-^{14}C]orotic acid (Table 6) showed that in yeast, as in the *E. coli* mutant, orotic acid was not an effective precursor of the pyrimidine residue of thiamine.

A pathway for which there is no direct evidence but which fits all the known facts is one that would involve β-methylaspartate instead of aspartate in the basic pathway, to give: carbamoyl-β-methyl-aspartate → 5-methylorotic acid → 5-methyluridine monophosphate. This is compatible with: (*a*) the failure to incorporate [^{14}C]orotic acid into the thiamine pyrimidine residue (Table 6; and Goldstein & Brown, 1963); (*b*) the much greater activity observed in the pyrimidine residue with [2-^{14}C]-acetate than with [1-^{14}C]acetate; this could be due to the incorporation of acetate into glutamate via the tricarboxylic acid cycle, which would involve much greater loss of C-1 than C-2 of acetate; glutamate could then be converted into β-methyl-aspartate by the vitamin B_{12} coenzyme-dependent isomerase (Barker, 1961); (*c*) the possible failure of incorporation of [^{14}C]formate into the 5-hydroxy-methyl group of the thiamine pyrimidine residue (David & Estramareix, 1961; and Table 1) [although David & Estramareix (1964) reported the presence of label from [^{14}C]formate in a derivative of the pyrimidine containing C-4, C-5, C-6 and the C attached to 3-5]. Although this may represent the general pathway of synthesis of the thiamine pyrimidine residue many details remain to be settled, e.g. the point at which the ribose phosphate is split off the base, because it is the free pyrimidine diphosphate that is condensed with the thiazole residue; also, the point at which the methyl group is inserted still remains unknown.

Generous grants from the Agricultural Research Council and Shell Research Ltd. allowed D. J. H. and D. B. J. to participate in this investigation. The paper was prepared during the tenure by T. W. G. of a National Science Foundation Senior Foreign Scientist Fellowship at the University of California, Davis.

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Metabolism of Iodomethane in the Rat

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(*Received* 20 *April* 1965)

1. The LD_{50} of iodomethane orally administered to rats is 76mg./kg. body wt. but repeated daily doses of 30–50mg./kg. body wt. are without effect. 2. Oral doses of iodomethane to rats are rapidly converted into *S*-methylglutathione in the liver, and *S*-methylglutathione is excreted in the bile. 3. The conjugation process has been reproduced *in vitro* and shown to be enzymically catalysed. 4. *S*-Methylglutathione is degraded to *S*-methylcysteine by kidney homogenates. 5. Only a small proportion of a dose of iodomethane or of *S*-methylcysteine is excreted as compounds related to methylmercapturic acid. 6. Paper chromatography of extracts of livers from normal and dosed rats supports the view that GSH is the predominant non-protein thiol present.

Chloro-, bromo- and iodo-methane are simple molecules having considerable toxic effects in man (for review see Oettingen, 1955). I have sought the reason for a lack of similar toxic effects in the rat. This paper describes the discovery of a mechanism in the rat for conjugating orally administered iodomethane with glutathione of liver and kidney. Degradative routes open to the formed *S*-methylglutathione have been considered: very little appears to be excreted as a mercapturic acid. Some of this work has been communicated briefly (Johnson, 1963).

The enzyme responsible for the conjugation is described in detail in the following paper (Johnson, 1966). Since this work was completed, descriptions have been published of work that identified urinary metabolites of some 2% of doses of *S*-methylcysteine and iodomethane given to rats (Barnsley, 1964; Barnsley & Young, 1965).

METHODS

Chemicals. Iodomethane was obtained from Hopkin and Williams Ltd. (Chadwell Heath, Essex) and was fractionally distilled before use. Suppliers of other chemicals are noted in parentheses: glycerol formal (Associated Chemicals Co., Leeds); iodoacetamide and *N*-ethylmaleimide (British Drug Houses Ltd., Poole, Dorset); *S*-methylglutathione (L. Light and Co. Ltd., Colnbrook, Bucks.); *S*-methylcysteine (California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.); GSH (Schwarz Bio-Research Inc., Mount Vernon, N.Y., U.S.A.); GSSG and *p*-chloromercuribenzoic acid (Sigma Chemical Co., St Louis, Mo., U.S.A.); 5,5-dithiobis-(2-nitrobenzoic acid) (Aldrich Chemical Co., Milwaukee, Wis., U.S.A.). All other compounds were of A.R. grade where available.

Animals. Female white rats (Porton strain) of about 200g. body wt. were usually used.

Administration of iodomethane. Oral administration was by stomach tube with a freshly prepared solution in glycerol formal. Intravenous injection was via tail vein with a solution in 90% (v/v) ethanol.

Trapping of exhaled iodomethane. A dosed rat was placed in a horizontal glass cylinder (3in. × 12in.) and dry air was drawn through at 800ml./min. Exhaled iodomethane was entrained and trapped at −25° in 75% (v/v) Cellosolve (200ml. in each of two Dreschel bottles connected in series). A 97% recovery of iodomethane was achieved when it was vaporized in the chamber at a steady rate after delivery by a slow injection device.

Collection and processing of urine. Rats were placed in metabolism cages, the outlets of which were lightly plugged with glass wool to prevent egress of faeces and food debris. Water was supplied to the rats in all experiments and food also where the collecting period was more than 24hr. Urine was collected in tubes cooled in ice–water in Dewar vessels; if not used immediately after a 20hr. collection period, the specimens were stored at −20°.

Had the expected metabolites been found in urine in high concentration, reasonable chromatographic separation might have been achieved without interference from salts and urea by using a very small sample of unprocessed urine. In practice, the small amount of ninhydrin-positive material present in 25µl. of urine was smeared badly on the developed chromatogram owing to the presence of salts in the urine. It was possible, however, to detect small quantities of material that reacted positively to the platinic iodide reagent (see under 'Paper chromatography' below) after they had been added to control urine and chromatographed. Detection of methylmercapturic acid and its sulphoxide and sulphone was simpler, a chloroform extract of acidified urine being used as the sample to be chromatographed as described by Thomson, Barnsley & Young (1963) for ethylmercapturic acid. Their procedure was modified slightly to avoid difficulties in extraction associated with

the presence of protein in the urine. Cold urine was acidified with $HClO_4$ (final concn. 8%, w/v) and frozen for 2hr. After thawing, the precipitate of protein and $KClO_4$ was removed by centrifuging and the supernatant was extracted twice with 3vol. of chloroform. Traces of non-volatile $HClO_4$ solution were removed from the extract by washing twice with small volumes of 0·1N-HCl. The extract was cooled to −20° and the ice crystals were removed by filtration at −5°. The dry chloroform extract was concentrated to about 2ml./original 50ml. of urine at 40° in a rotary evaporator and the resulting solution was ready for chromatography or for acid hydrolysis. Methylmercapturic acid does not respond to the ninhydrin test because of its acetylated amino group, and the sulphoxide responds poorly to the platinic iodide test. Both these compounds, however, can be converted into *S*-methylcysteine by boiling under reflux with an equal volume of conc. HCl (Thomson *et al.* 1963) or with HI (Morris & Thompson, 1956). Urine samples were treated directly and chloroform extracts were evaporated to near dryness and then taken up in a small volume of 50% (v/v) ethanol before acid treatment. Conc. HBr was preferred to HCl because of the lower likelihood of loss of *S*-methylcysteine by conversion into alanine with concomitant loss of thiol (Grodsky, Carbone & Fanska, 1959). Direct HBr treatment of urine suffered from interference by hydrolysis of protein and also by the production of a red insoluble pigment that darkened as heating continued: little appears to be known of the nature of this material.

A more thorough, though more laborious, treatment of urine before chromatography consisted in deproteinization with $HClO_4$, neutralization with KOH and separation of amino acids into basic, neutral and acidic fractions by passage through a Dowex 1 (CO_3^{2-} form) column followed by elution with 0·1N-HCl (Gaitonde, 1961). The acidic and neutral fractions were concentrated to 1ml. each/original 10ml. of urine and used for paper chromatography: methylmercapturic acid and its derivatives would be expected in the acidic fraction, and *S*-methylcysteine and its sulphoxide and sulphone in the neutral fraction.

Collection of bile. A fine polythene cannula was inserted into the bile duct of a large (240–270g.) female rat under ether anaesthesia and anchored so that, when the animal was placed in a wire-mesh restraining cage, bile could be collected in a cooled graduated tube without interference from the rat (I thank Dr J. M. Barnes, who performed the operations). The bile was stored overnight at −20° and used directly for paper chromatography: considerable care was needed in applying the sample to prevent it from spreading unduly.

Deproteinization of tissues. A 1ml. blood sample was lysed in 2ml. of water containing a pinch of saponin: 3ml. of 3% (w/v) metaphosphoric acid (saturated with NaCl) was added and mixed thoroughly. After 10min. the suspension was centrifuged to remove solids. All other tissues were homogenized in ethanol–24mM-KH_2PO_4 solution, pH5·5 (3:1, v/v), and centrifuged to remove protein. Provided that not more than 2g. of liver was homogenized in 15ml., the recovery of GSH was 85% and that of *S*-methylglutathione was 95%. Before paper chromatography, deproteinized extracts were treated anaerobically with a 70% excess of iodoacetamide or *p*-chloromercuribenzoate at pH10 for 10min. at 20° to react with free thiol groups and then rapidly concentrated fivefold in a rotary vacuum evaporator at 30°.

Paper chromatography. Descending chromatography was performed with 10–25μl. samples for 16hr. (overnight) on Whatman no. 1 paper sheets at 25°. To measure R_F values, only a limited volume of developing solvent was used so that it should not run off the end during the period, and this led to an increase in R_F values when the solvent was butan-1-ol–acetic acid–water: this may have been due to insufficient saturation of the tank atmosphere with vapour and an alteration of solvent composition at the solvent front. Papers were dried in air before detection procedures were carried out. Ninhydrin was applied by dipping the paper into 0·25% solution in acetone, air-drying and heating at 55° for 30min. The platinic iodide and nitroprusside dip reagents were those of Toennies & Kolb (1951). Platinic iodide may be superimposed on papers that have been treated with ninhydrin and subsequently bleached by dipping into a 1% (v/v) solution of conc. HCl in acetone and air-drying, but there is some loss of sensitivity. The platinic iodide reagent detected a minimum of about 2μg. of *S*-methylcysteine, whereas 5μg. was needed for a positive ninhydrin reaction.

The $AgNO_3$–$K_2Cr_2O_7$ double spray reagent of Knight & Young (1958) for thiol derivatives was difficult to apply without getting runny smears of the aqueous reagents on the paper. It was found possible to convert both reagents into solutions containing a high percentage of acetone in which chromatograms could be dipped successively with only 2–3min. drying between dips. Either reagent could be applied first. Stock solutions were: (1) $AgNO_3$ (8·5g.) in water (125ml.) and acetone (375ml.); this was stable when stored in a dark bottle and was diluted with 3vol. of acetone before use; (2) $K_2Cr_2O_7$ (7·35g.) in water (250ml.) and acetic acid (250ml.), which was diluted with 7vol. of acetone before use. A 0·1μmole portion of cysteine and glutathione and their *S*-methyl and *S*-(*N*-ethylmaleimide) derivatives showed as pale-yellow to yellow–orange spots on a medium-orange background. However, the platinic iodide reagent was somewhat more sensitive and was the preferred reagent.

The following solvents were used: *A*, butan-1-ol–acetic acid–water (11:4:5, by vol.); *B*, phenol–water (4:1, w/v); *C*, phenol–aq. NH_3 [solvent *B* plus 0·5% (v/v) of aq. NH_3 (sp.gr. 0·88)]; *D*, phenol–propan-2-ol–water–acetic acid (60:25:15:2, by vol.); *E*, butan-1-ol–pyridine–3N-NH_3 (4:3:3, by vol.).

Dried spots at the origin were sometimes treated with 0·05ml. of 30% (v/v) H_2O_2 plus 0·05ml. of 0·1% ammonium molybdate to convert thiol derivatives into sulphones (Dent, 1948).

Assay of thiol groups. Total tissue thiol was measured in clear supernatants of sucrose homogenates by reaction with *p*-chloromercuribenzoate and estimation of the excess of reagent with dithizone (Fridovich & Handler, 1957). Non-protein thiol content was measured in deproteinized tissue extracts with 5,5′-dithiobis-(2-nitrobenzoic acid) as described by Beutler, Duron & Kelly (1963) or with nitroprusside (Grunert & Philips, 1951). Both reagents gave similar results with pure standards and tissue extracts but the former reagent is simpler, more stable, more sensitive and yields a chromophore stable in dim light.

Assay of iodomethane. This used the γ-(4-nitrobenzyl)-pyridine reagent as described by Johnson (1966).

Enzymic degredation of S-methylglutathione. *S*-Methylglutathione (6–8mM) was incubated at 37° for 60min. with

cell-free liver or kidney homogenate (up to 18mg. of tissue/ml. of reaction medium: 50mM-triethanolamine hydrochloride buffer, pH7·5). The reaction was halted by heating at 100° for 2min. and the precipitate was removed by centrifuging; 25μl. portions of the supernatant were used for chromatography.

RESULTS AND DISCUSSION

Toxicity. The LD_{50} for iodomethane orally administered in glycerol formal carrier was 76mg./kg. body wt. (determined as described by Weil, 1952). Rats took 5–10hr. to die and in subsequent metabolic studies they were usually killed well before signs of poisoning were apparent so that effects were unlikely to be due to a general failure of metabolism in a dying animal. In spite of its low boiling point (42°) only 1% of an LD_{50} dose of iodomethane was found in exhaled air during 30min. after dosing. Miller & Haggard (1943) found little exhalation of the even more volatile bromomethane (b.p. 4°), although a high proportion of a dose of bromoethane (b.p. 38°) was exhaled. It seems that the iodomethane was rapidly metabolized to non-volatile products.

Oral doses of 30–50mg. of iodomethane/kg. body wt. 5 days a week for a month produced no toxic effects. This also suggested rapid metabolism to products of lower toxicity.

Primary reaction. Reaction of iodomethane with tissue GSH was considered since orally administered bromobutane has been shown to lower the GSH content of rat liver *in vivo* (Barnes, James & Wood, 1959). The effect of oral administration of iodomethane on the non-protein thiol content of several tissues of the rat is shown in Table 1. Only small effects were observed on the concentration in brain and blood, but a massive fall in the concentration in liver occurred and this was maximal at from 30 to 120min. after dosage. At the highest doses a fall in the kidney non-protein thiol content was also seen but only after depletion was nearly complete in the liver. A small (not more than 20%) and varied depressive effect was also observed on liver protein thiol contents after a dose of 75mg./kg. body wt. to a few animals; the accuracy of this observation is uncertain.

A compound indistinguishable from *S*-methylglutathione by chromatography in solvents *A* and *B* was found in deproteinized extracts of rat liver

Table 1. *Effect of oral dosage of iodomethane on the non-protein thiol content of rat tissues*

Female rats (about 200g.) were dosed between 11 a.m. and noon with iodomethane dissolved in glycerol formal so that each rat received 0·2ml. of glycerol formal/200g. body wt. Control rats usually received a similar dose of glycerol formal but this produced no alteration compared with undosed rats. Extraction of tissues and assay were as described in the Methods section. The absolute control values are given as means ± S.D.

Dose (mg./kg. body wt.)	Time after dosage (min.)	Non-protein thiol content (% of control)			
		Liver	Kidney	Brain	Blood
None (control)	0–180	100 [5·2±0·6 μmoles/g. wet wt. (25 rats)]	100 [3·3±0·5 μmoles/g. wet wt. (11 rats)]	100 [1·7±0·1 μmoles/g. wet wt. (4 rats)]	100 [1·13±0·12 μmoles/ml. (6 rats)]
50	15	71	—	—	—
	30	48	—	—	—
	45	61	—	—	—
	60–70	59, 53, 54	—	—	—
	120–140	63, 45, 73	—	—	—
	180	61	—	—	—
	240	68	—	—	—
75	15	53	92	—	—
	30	11	87	—	—
	50–70	26, 15 23, 17	80, 82	71	100
	90	17	83	—	—
	120	19	68	89	100
	180	44, 48	—	—	—
100	45	<10	59	63	—
	120	<10	6,10	90	86, 79
	180*	<10	16, 39	—	—

* Rats very ill.

Table 2. *R_F values of compounds detected in liver extracts from control and dosed rats*

Samples were prepared as described in the Methods section. The 25μl. portions (about 30μg. of GSH) were chromatographed in solvents *A* and *B* and examined with the platinic iodide and ninhydrin reagents: all the spots tabulated responded positively to both reagents. With solvent *A* only 60ml. of solvent was used (see the Methods section). Values in parentheses refer to the platinic iodide-tested papers. Traces of GSSG (R_F 0·10 in solvent *A* and R_F 0·15 in solvent *B*) were found both with standards and with control liver extracts but not with liver extracts from dosed animals. This may indicate oxidation during chromatography after incomplete protection of the thiol group. Standard R_F values are quoted for samples run in the presence of liver extract, but are not significantly different from normal. From extracts further ninhydrin-positive spots were obtained that were similar in size and distribution whether the extract came from normal or dosed animals. The most prominent had R_F values: in solvent *A*, 0·26 and 0·36; in solvent *B*, 0·30 and 0·59.

Compound	R_F in solvent *A*	R_F in solvent *B*
Extract treated with iodoacetamide		
Glutathione standard	0·20 (0·21)	0·41 (0·39)
S-Methylglutathione standard	0·41 (0·42)	0·54 (0·53)
Control liver	0·22 (0·21)	0·41 (0·39)
Dosed liver	0·41 (0·42)	0·54 (0·53)
Extract treated with *p*-chloromercuribenzoate		
Glutathione standard	0·49 (0·47)	0·29 (0·29)
S-Methylglutathione standard	0·44 (0·45)	0·47 (0·50)
Control liver	0·49 (0·47)	0·29 (0·29)
Dosed liver	0·44 (0·47)	0·47 (0·50)

Table 3. *R_F values of compounds detected in bile from control and dosed rats*

Bile samples were obtained as described in the Methods section. Portions (25μl.) (some containing added reference compounds) were chromatographed in solvents *A* and *B*. Further samples were also treated at the origin with H_2O_2–molybdate before chromatography. With solvent *A* only 60ml. of solvent used (see the Methods section). All spots recorded were ninhydrin-positive; * signifies that the spot was also platinic iodide-positive. Both *S*-methylcysteine and glutamic acid were less easily detected with ninhydrin after H_2O_2 treatment.

Compound	R_F in solvent *A*		R_F in solvent *B*
	No. H_2O_2	H_2O_2-treated	H_2O_2-treated
Standards			
Glycine	0·31	0·31	0·42
Glutamic acid	0·36	0·36	0·33
S-Methylcysteine	*0·53	0·25	0·47
S-Methylglutathione	*0·41	0·23	0·39
Glutathione	*0·39	—	—
Control bile	← No substantial spot →		
Bile from dosed animals			
Substantial spots	*0·41	0·23	0·39
	*0·51		
Traces	0·31	0·25	0·43
	0·36	0·33	0·47
	—	0·39	—

prepared 40min. after the administration of iodomethane (90mg./kg. body wt.) to rats (Table 2). This sole product replaced the only compound reacting positively to both ninhydrin and platinic iodide reagents that was detected in significant amounts in extracts of normal liver and that was indistinguishable from GSH (Table 2). Only traces of GSSG were found in extracts of liver of both normal and dosed rats. The results indicate that orally administered iodomethane is rapidly converted into *S*-methylglutathione in the liver.

That the methylation reaction was enzymically catalysed is shown by the following observations: incubation of iodomethane with GSH in the presence of homogenates of liver, kidney and adrenal tissue led to a rapid loss of iodomethane; negligible reaction was observed in the absence of GSH or with homogenates of blood, brain, heart or spleen, nor did it occur with liver homogenates heated at 70° for 4min. to denature the protein. Full details of this experiment and further studies of the responsible enzyme (glutathione *S*-alkyltransferase) are given in the following paper (Johnson, 1966). The reaction of many other alkyl compounds with liver GSH *in vivo* is described elsewhere (Johnson, 1965).

Biliary excretion of S-*methylglutathione.* In two experiments about 22 and 28% respectively of oral doses of iodomethane (50mg./kg. body wt.) were detected mainly as *S*-methylglutathione with traces of *S*-methylcysteine in bile collected from cannulated rats for 6hr. after dosing (Table 3).

Degradation of S-*methylglutathione.* *S*-Methylglutathione was not measurably degraded by liver homogenate under the stated conditions (see the Methods section), but was degraded by kidney homogenate at a rate of 3·5m-moles/g. of tissue/hr. to yield products identified as glycine, glutamic acid and *S*-methylcysteine by chromatography in solvents *A* (unlimited volume) and *E* (R_F values were: in solvent *A*, 0·23, 0·28 and 0·38 respectively; in solvent *E*, 0·29, 0·20 and 0·45 respectively) and by staining with ninhydrin and platinic iodide reagents. The rate of reaction was not markedly influenced by the presence of EDTA (10mM) in the medium. This contrasts with Binkley's (1961) finding that the enzyme of pig kidney catalysing the

second stage of GSH degradation to amino acids is completely inhibited by EDTA.

It is clear from the present work that the rat can easily degrade *S*-methylglutathione, but this may only occur if the material excreted in the bile is reabsorbed and circulated to the kidney.

Further metabolism of S-methylcysteine. After rats had been dosed orally with iodomethane (50mg./kg. body wt.) or intravenously with *S*-methylcysteine (50mg./kg. body wt.), chromatographic investigation of 25μl. portions of urine or of chloroform extracts of acidified urine (see the Methods section) revealed no compounds responding positively to the platinic iodide reagent, but after treatment of the urine with hydrobromic acid (see the Methods section) traces were seen of a compound positive to the ninhydrin and platinic iodide reagents and having R_F 0·52 in solvent *A* (limited volume) and R_F 0·43 in solvent *D*. This was probably *S*-methylcysteine (R_F values of authentic samples 0·52 and 0·45 respectively) derived from methylmercapturic acid and its sulphoxide by hydrolysis and reduction, but the quantity detected was so small (equivalent to less than 2% of the dose) that the investigation was not pursued. Barnsley (1964) and Barnsley & Young (1965) have since confirmed the very small total urinary excretion of metabolites of iodomethane and *S*-methylcysteine and have demonstrated that, besides compounds related to methylmercapturic acid, methylthioacetic acid and its glycine conjugate are present in the urine.

GENERAL DISCUSSION

Excretion of iodomethane analogues. The urine of rats contains higher proportions of recognized metabolites after the administration of higher alkyl halides, alkylcysteines and other compounds that are probably metabolized via *S*-alkylated cysteine than after iodomethane (Roberts & Warwick, 1958; Thomson, Maw & Young, 1958; Bray & James, 1958; Greenby & Young, 1960; Thomson *et al.* 1963; Barnsley, Thomson & Young, 1964; Bray, Caygill, James & Wood, 1964). However, Horner & Kuchinskas (1959) showed that 34–37% of a large dose (115mg./kg. body wt.) of *S*-methylcysteine administered intraperitoneally to rats was exhaled as carbon dioxide within 24hr. Binkley (1950) showed that *S*-methylcysteine is readily degraded to a volatile thiol (? methanethiol) by a cysteine desulphydrase preparation from rat liver, and a microsomal *S*-demethylase capable of attacking *S*-methylcysteine has been described by Mazel, Henderson & Axelrod (1964). Barnsley (1964) has shown that the conversion of *S*-methylcysteine into methylthioacetic acid occurs in the rat: this can be oxidized in an unknown fashion to sulphate (Maw, 1953) or conjugated to glycine and excreted. It seems possible that higher homologues of *S*-methylcysteine are less readily metabolized by these systems and remain therefore to be *N*-acetylated and excreted in the urine as mercapturic acids.

Fate of iodomethane. A large proportion of the total dose of iodomethane is accounted for by reaction with GSH *in vivo*. By using the values of Table 1 and the fact that GSH turns over rapidly in liver [$t_{\frac{1}{2}}$ = 3–4hr. (Anderson & Mosher, 1951; Kalser & Beck, 1963)], it is possible to calculate that about 45–50% of doses of 50–75mg. of iodomethane/kg. body wt. given to a 200g. rat (liver wt. say 8g.) is accounted for by reaction with GSH in 1½hr. More is probably lost in the slight reaction observed with protein thiol groups, and a considerable amount is probably lost in a damaging reaction with the stomach wall since much accumulation of fluid in the stomach is observed after dosage.

GSH concentrations in liver rapidly return to normal after depletion by iodomethane. Liver homogenate did not appreciably demethylate *S*-methylglutathione *in vitro* and the compound is excreted in bile, so that the return to normal GSH concentrations in the liver is presumably due to fresh synthesis: this restoration of GSH concentration explains the lack of toxicity of repeated daily doses each equal to half the LD_{50}.

It is not clear whether the conjugation with GSH is such an effective mechanism for protection against iodomethane entering the body by routes other than oral. I have found evidence (unpublished work) of altered carbohydrate metabolism in brain slices taken from rats exposed to high concentrations (10–20mg./l.) of the vapour of bromomethane or iodomethane for 1hr. and these effects can be mimicked by the addition of iodomethane (3mM) to normal brain slices *in vitro*. It is clear therefore that some of the vapour reaches the brain unchanged but I have not measured the concentrations of GSH in tissues after such exposures. After the intravenous injection of iodomethane to rats a rapid but moderate fall in the GSH concentrations in blood, brain, liver and kidney is observed (M. K. Johnson, unpublished work), and this suggests that at the very high initial concentration rapid non-enzymic reaction occurs with GSH without aid from glutathione *S*-alkyltransferase, which has not been observed in blood or brain. Non-enzymic reaction probably occurs with other tissue constituents also.

Liver glutathione. When deproteinized extracts of normal rat liver were treated with thiol reagents and chromatographed, only the appropriate glutathione derivative could be found (Table 2). This has been confirmed with three other chromatography solvents and also after *N*-ethylmaleimide

treatment of the extract (M. K. Johnson, unpublished work). Although the extraction is only about 85% efficient for GSH (see the Methods section), it seems likely that GSH is the predominant non-protein thiol of rat liver. This view contrasts with that of Calcutt & Doxey (1962), but agrees with reports by Davidson & Hird (1964) and Owens & Belcher (1965), who used glutathione reductase and glyoxalase for the specific assay of tissue glutathione.

Retention of glutathione in liver cells. A concentration gradient exists for GSH between liver cells, where it abounds, and plasma, which contains very little. The contrast between the retention of GSH in the liver and the rapid excretion of *S*-methylglutathione suggests that some positive process is needed to retain the former and that the thiol group is implicated.

I thank Dr W. N. Aldridge for much stimulating discussion and encouragement and Mrs M. A. Thomas for skilled technical assistance. Most of the work described here has been included in a Thesis for the Ph.D. award of the University of London.

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Studies on Glutathione *S*-Alkyltransferase of the Rat

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(*Received* 20 *April* 1965)

1. A rat-liver enzyme catalysing the *S*-alkylation of glutathione by iodomethane and various other alkyl compounds has been identified and partially purified; its stability, specificity and response to inhibitors and activators and to changes in reaction pH have been studied. 2. The enzyme is distinct from glutathione *S*-aryltransferase, but both enzymes respond similarly to various inhibitors. 3. A similar enzyme has been found in the kidney and adrenal of rat and in the liver and kidney of numerous species. 4. The identity and the physiological role of the enzyme are discussed.

In the preceding paper (Johnson, 1966) the rapid conversion of rat-liver GSH into *S*-methylglutathione after an oral dose of iodomethane was reported and brief evidence was stated for the fact that this reaction is enzymically catalysed. The present paper is devoted to a study of the enzyme. Some of this work has been briefly reported (Johnson, 1963).

METHODS

Materials. Besides those listed in the accompanying paper (Johnson, 1966) the following were obtained from the sources noted: bromomethane (May and Baker Ltd., Dagenham, Essex); all other alkyl halides (Hopkin and Williams Ltd., Chadwell Heath, Essex): they were fractionally distilled before use; chloroacetic acid, chloroacetamide and iodoacetic acid (British Drug Houses Ltd., Poole, Dorset); chloroacetaldehyde [aq. 40% (v/v) solution], chloroacetonitrile, 2-bromopropionic acid, 3-bromopropionic acid, 2-mercaptopropionic acid, 2-mercaptoethylamine, β-propiolactone and 3,4-dichloronitrobenzene (L. Light and Co. Ltd., Colnbrook, Bucks.); ^{14}C-labelled compounds (The Radiochemical Centre, Amersham, Bucks.); ^{14}C-labelled bromoethane was synthesized from labelled ethanol by a small-scale version of the standard preparation described by Vogel (1948): 5g. of bromoethane (330μc) was obtained from 4ml. of ethanol (500μc).

Enzyme assays. Unless specifically noted, assays were at pH7·0 to obtain low rates of non-enzymic reaction. GSH solutions normally contained EDTA (2mM) and were purged with N_2 before use: even at pH9 no loss of free thiol occurred in such solutions during 1hr. at 20°. Alkyl halides were added from stock solutions in acetone, which did not influence the reaction at concentrations below 3% (v/v).

(*a*) By disappearance of iodomethane. Iodomethane (about 0·5mM) and GSH (1–2mM) were incubated in 50mM-phosphate buffer, pH7·0, or 50mM-triethanolamine hydrochloride buffer, pH7·0, with tissue homogenate at 37°. The reaction was stopped by the addition of cold $HClO_4$ (final concn. 6%, w/v). Residual iodomethane was extracted from the deproteinized solution and assayed by a modification of the method of Epstein, Rosenthal & Ess (1955). Results were rather variable but the method was useful for screening purposes.

(*b*) By disappearance of thiol group. The use of the 5,5′-dithiobis-(2-nitrobenzoic acid) reagent and its sensitivity has been described in the preceding paper (Johnson, 1966). GSH (0·5–2mM) and alkyl halide (0·5–6mM) were incubated with enzyme preparations at 25° in 66mM-phosphate buffer, pH7·0, containing EDTA (2mM). Samples were withdrawn and quenched each minute up to the sixth minute. The manner of quenching depended on the purity of enzyme. In experiments with partially purified enzyme, it was sufficient to transfer rapidly a sample (0·16–0·45ml., accurately measured with a syringe pipette) of the reaction mixture to a tube containing 6ml. of 0·3M-Na_2HPO_4, pH7·7, and 1ml. of 5,5′-dithiobis-(2-nitrobenzoic acid) reagent (0·04% solution in 0·3M-Na_2HPO_4, pH7·7). The tube was stoppered and contents were mixed quickly. The reaction was stopped instantly by the conversion of residual GSH into a mixed disulphide with liberation of the yellow reagent thiol anion: the extinction was read at 410mμ (the soluble protein in the mixture had no effect). When tissue extracts were used to supply enzyme, the reaction mixture was opaque and not suitable for direct assay; 2ml. samples were removed and blown into 4ml. of cold 3% (w/v) sulphosalicylic acid to stop the reaction. Protein was removed by centrifuging and portions (0·5–2ml.) of the deproteinized supernatant were assayed with the reagent as above. Some reactive thiol groups of proteins in rat-liver supernatant reacted with the reagent and contributed an appreciable blank. This blank declined during the course of reaction as these groups were rapidly attacked by iodomethane; it was usually found that a steady rate of reaction was obtained after an apparent surge during the first 2min. while this non-enzymic attack on protein thiol groups was being measured in addition to the transferase reaction: the protein of stock purified enzyme (see below) produced no appreciable blank. At the concentrations of GSH usually studied, the removal of GSH equivalent to an extinction change of more than 0·08 (about 15% of the

total) significantly decreased the rate of reaction: the method was therefore of greatest use in qualitative and comparative experiments rather than in precise kinetic determinations.

(*c*) By production of H^+ ion. The production of H^+ ion in 16ml. of solution containing GSH (6mM) and alkyl halide (2–6mM) plus purified enzyme in triethanolamine hydrochloride (0·5mM) and EDTA (0·2mM) was followed at pH7·0 and 20° with the Radiometer pH-stat.

(*d*) By production of I^- ion. This method has not been described previously. Street (1960) described an assay of serum bromide based on a similar principle. A 25ml. reaction mixture containing GSH and iodomethane (both 6mM) and tissue was incubated at 25° for up to 16min. in 66mM-phosphate buffer, pH7–8, or 66mM-triethanolamine buffer, pH7–8, both containing EDTA (1–2mM). Samples (4ml.) were taken every 2min. and quenched and treated according to their protein content; when it was low, as in assays with iodomethane and purified enzyme, the sample was blown into a cold (10°) mixture of 1ml. of 10% (w/v) $Na_2Cr_2O_7$ solution in 6N-H_2SO_4 plus 4ml. of cyclohexane in a centrifuge tube. The stoppered tube was shaken vigorously for 1min. to oxidize I^- ion and to extract I_2 from the deproteinized solution, and then centrifuged at 1500*g* for 20min. to separate the phases. The small volume of protein remained at the interface. The extinction of a portion of the upper organic phase was measured at 520mμ. The I_2 solution produced from 4ml. of 1mM-KI solution under these conditions had an extinction of 0·42 (0·405–0·43 in several experiments). With larger quantities of protein, phase separation was not so clear and loss of I_2 occurred by adsorption; it was necessary to deproteinize a 4ml. reaction sample with 1ml. of 60% (v/v) $HClO_4$ and to assay I^- ion in a 4ml. portion of protein-free supernatant obtained after centrifuging. Residual iodomethane or iodoethane in the reaction mixture yielded no measurable I_2. The $Na_2Cr_2O_7$ reagent was stable indefinitely. Reaction rates linear with time and enzyme concentration were obtained when purified enzyme was used. Because of the method's low sensitivity, high substrate concentrations were used and appreciable non-enzymic blanks were incurred during the comparatively long reaction periods studied. The method was not suitable for assays in which Br^- ion was liberated because the extinction coefficient of Br_2 solution is only about one-fifth that of I_2.

(*e*) By production of non-volatile radioactive glutathione derivative. This method was only used with purified enzyme preparations and required ^{14}C-labelled alkyl halide. Tracers were diluted with stock concentrated solutions of alkyl halide in acetone and stored at 0°. The reaction was studied at pH7·0 and 25° in 66mM-phosphate buffer containing EDTA (2mM). GSH (0·4–3mM), alkyl halide (0·4–6mM; up to 15μc) and purified enzyme were present. In individual experiments the total volume was sometimes varied to obtain a range of final concentrations from a fixed quantity of alkyl halide added from stock; this led to some anomalous results and it seemed possible that losses of alkyl halide by evaporation varied according to the surface/volume ratio. Subsequent work used a fixed reaction volume (usually 6 or 8ml.) in each experiment and a range of radioactive stock solutions was prepared to supply different final substrate concentrations. Four samples (0·5ml.) were withdrawn with an accurate syringe pipette at fixed intervals (usually 1min.) during the reaction and quenched immediately by blowing them into 0·5ml. of 1% (v/v) Br_2 solution in methanol, which oxidized GSH and denatured protein; the quenching was performed in scintillation-counter vials. The Br_2, water, methanol and unchanged volatile substrate were evaporated from the vial in a fume-cupboard by blowing air over the surface for several hours. The non-volatile solid residue (except protein) was dissolved in hot water (0·5ml.), and 14ml. of a scintillation medium capable of holding water (the XDC medium of Bruno & Christian, 1961) was added. The radioactivity of the sample was measured by a 10min. count in a Packard series 3000 Tri-Carb counter. Under optimum conditions (25% gain, window set to 100–1000v), the efficiency of counting was 70–74% and was not influenced by the traces of insoluble salts and denatured protein in the mixture. The rate of reaction was determined from the slope of the line through points on a graph plotted of sample counts against time of sampling: good linear plots were obtained but these did not pass through the origin (Fig. 1). One cause was the presence of traces of non-volatile material in the tracer supplied (particularly with iodoethane): however, with some but not all enzyme preparations the zero blank was considerably greater with alkyl halide incubated with both GSH and enzyme than the sum of the blanks from halide plus GSH and halide plus enzyme. It seemed probable that GSH interacted with proteins (possibly by reduction of disulphides) to form in them reactive chemical groups available for alkylation. Provided that substrate of reasonable specific activity was used, it was not necessary to utilize more than 5% of the substrate. There was a linear relationship of rate to tissue concentration. When reaction times were brief, blanks were negligible (less than 2%) except with iodomethane, where the blank was up to 10% of the total reaction at high substrate concentration. Non-enzymic rates were usually determined over longer periods

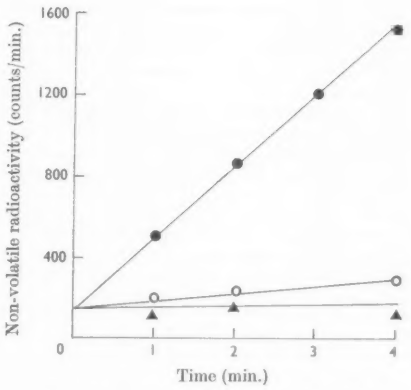


Fig. 1. Enzyme assay method *e*: rate of production of non-volatile radioactivity. The assay was carried out at pH7·0 and 25° as described in the text; the concentration of both substrates was 2mM, the specific activity of iodomethane being 41μc/m-mole. ▲, Enzyme+iodomethane; ○, GSH+iodomethane; ●, enzyme+GSH+iodomethane.

(8min. for iodomethane; up to 2hr. for the others). This assay method was undoubtedly the most accurate of the five and was used for the determination of kinetic constants whenever labelled substrates were available.

Enzyme purification. Rat liver (6g.) was homogenized with a smooth Perspex pestle (Aldridge, Emery & Street, 1960) in ice-cold unbuffered 2% (w/v) NaCl solution (23ml.), and 12 combined homogenates (fraction A; see Table 3) were centrifuged at 2° in the angle 30 head of the Spinco model L ultracentrifuge at 60000*g* for 30min. The cloudy supernatants were combined and diluted to 320ml. with 2% NaCl containing concentrated triethanolamine hydrochloride and hydroxylamine–acetate buffers to give a final 10mM concentration of each buffer species. The pH at this stage was 6·3–6·8 in different preparations. The supernatant (fraction B) was kept at 2° in an ethanol–solid CO_2 bath and stirred magnetically: 64ml. of 90% (v/v) ethanol precooled to −5° was added rapidly to give a final ethanol concentration of 15% (v/v) and the pH was adjusted to read 6·0 at 2°. No attempt was made to compensate for the effect of low temperature on the performance of the pH-meter electrodes. It was found throughout these fractionations that the pH of the suspensions altered noticeably in the first few minutes after the addition of a precipitation reagent, and checking and readjustment of pH was performed as a routine 5min. after any pH adjustment: no further adjustment was then needed. The pH adjustments were made with solutions of acetic acid or triethanolamine. After 20min. at 0–2°, the grey precipitate was removed by centrifuging at 5000*g* for 5min. and discarded. A 0·2M-$ZnSO_4$ solution was added to the deep-red supernatant (fraction C) to bring the concentration to 2·2mM. After the mixture had been stirred at 0–2° and pH6·0 for 20min., the red precipitate was removed by centrifuging at 5000*g* for 5min. and discarded. A 0·2M-$ZnSO_4$ solution was added to the pale-golden-yellow supernatant (fraction D) to increase the concentration by 12·8mM (i.e. final concn. 15mM if the Zn^{2+} removed with the first precipitate is ignored). After the mixture had been stirred at 0–2° and pH6·2 for 20min., the brown precipitate containing most of the activity was removed by centrifuging at 5000*g* for 5min. The white opalescent supernatant was discarded. The pellet was suspended in 2% NaCl made 10mM with respect to each of hydroxylamine–acetate and triethanolamine buffers, pH7·0, and containing EDTA (4mM) (referred to as NaCl/H/T/V) and rapidly dissolved by the addition of 8ml. of 0·2M-EDTA (slow dissolution could be achieved with about 5ml.): the orange–yellow solution (fraction E) was diluted with NaCl/H/T/V/ to 178ml. at pH7·0. Then $(NH_4)_2SO_4$ (52·2g.) was added to bring the solution to 47% saturation and the suspension was stirred at 0–2° and pH7·0 for 30min. The tan-coloured precipitate was removed by centrifuging at 5000*g* for 10min. and discarded. The pale-yellow solution (199ml.) was brought to 60% saturation with $(NH_4)_2SO_4$ (an extra 16·6g.) and stirred at 0–2° and pH7·0 for 30min. The active precipitate (fraction F) was removed by centrifuging at 5000*g* for 10min. and dissolved in NaCl/H/T/V, which was adjusted to a volume of 25ml. at pH7·0. The solution was pale yellow. For comparative assay all precipitates were dissolved in NaCl/H/T/V (with extra EDTA as needed) and all solutions were adjusted to pH7·0. For storage of the active fraction F, $(NH_4)_2SO_4$ was added to give 70% saturation without adjustment of pH (which was usually about 6·5): the white suspension (fraction G) was stored at −5° and was stable for many weeks. Samples were usually prepared for study by centrifuging down the protein and redissolving the pellet in suitable buffer: much of the remaining glutathione aryltransferase activity of fraction G remained in the supernatant and was discarded, so that the final ratio of activity towards the two substrates was about 25–30 compared with the value of 10 achieved with fraction F (Table 3). Fractions obtained in the purification process were assayed by method (*b*) and a deproteinization step was included for samples up to the end of the $ZnSO_4$ fractionation, but not thereafter. Protein was determined by the biuret method (Robinson & Hogden, 1940) on early fractions and by the extinction at 280mμ in later steps. As performed the biuret method gave an extinction of 1·0/38mg. of protein in a final 11ml. (calibrated with bovine serum albumin) and E_{280} gave an extinction 1·0/2–2·2mg. in 3ml. (roughly calibrated by comparison with biuret samples).

Chromatography. Paper chromatography with solvent descending for 16hr. was with butan-1-ol–acetic acid–water (11:4:5, by vol.). Dried papers were stained by dipping in ninhydrin (0·25% in acetone) and heating at 55° for 30min.

RESULTS

The disappearance of iodomethane was catalysed by homogenates of liver, kidney and adrenal of the rat in the presence of GSH (Table 1). The enzymic nature of the reaction is shown by the tissue specificity and the lack of reaction in the absence of GSH or when denatured liver homogenate was used (Table 1). That GSH is a second substrate and not a cofactor was shown by incubating purified enzyme with both compounds (6mM). Consumption of thiol and production of I^- ion was equivalent [26·8 and 26·9 μmoles respectively in 6min. (assays

Table 1. *Enzymically catalysed reaction of glutathione and iodomethane*

Tissue was homogenized in the balanced saline medium of Rodnight & McIlwain (1954) diluted threefold with 2% NaCl (high ionic strength stabilizes the enzyme). The disappearance of iodomethane was followed by assay (*a*). * Signifies that the loss was not significant.

Homogenate	Iodomethane lost (μmoles/min./g. of tissue)
Liver	1·7
Liver (heated at 70° for 4min.)	<0·25*
Liver (GSH omitted from assay)	<0·25*
Kidney	1·4
Adrenal	0·6
Heart	<0·25*
Lung	<0·25*
Spleen	<0·25*
Brain	<0·25*
Blood	<0·25*

b and *d*)], and a single product was found corresponding to authentic *S*-methylglutathione on chromatographic investigation: this completely replaced the GSH spot if the reaction was allowed to run to completion (R_F of GSH 0·38; R_F of *S*-methylglutathione 0·42). The non-enzymic reaction was about 8% of the total.

Subcellular distribution. Differential centrifugation of liver and kidney homogenates in 0·3M-sucrose showed that about 80% of the activity was in the supernatant fraction (assay *a*). Supernatants obtained from homogenates prepared in 2% sodium chloride solution and centrifuged at 60000*g* for 30min. were highly active (85% of the whole homogenate activity in one sample assayed with the pH-stat) and were used for many studies, as the starting material for purification of enzyme, and also as the standard preparation for inter-species comparisons. Such supernatants are referred to below as 'extracts'.

Cofactors. No diffusible cofactors were found. Dialysis of liver extracts against deionized water for 5hr. at 2° caused a heavy flocculent precipitate to form, and only 28% of the activity was recovered from the soluble protein and almost none from the precipitate, which was mostly insoluble in 2% sodium chloride. A similar effect was observed with kidney extract. This loss of activity was due to denaturation of the protein in the solution of low ionic strength rather than to loss of cofactor, since dialysis for the same time against 2% sodium chloride solution in deionized water led to only a slight precipitation of protein and retention of 72–95% of the activity in solution in several experiments.

Assayed rates were unaltered by the presence or absence in the medium of EDTA (2mM), which was normally added to protect GSH against oxidation catalysed by heavy-metal ions.

Stability. The activity of supernatants from sucrose homogenates was extremely variable from experiment to experiment and declined rapidly at 2°. Supernatants in 2% sodium chloride appeared fairly stable for 5hr. at 2°, but both supernatants lost all activity if stored for 18hr. at 2° and both retained full activity when stored at −20° for 48hr. Activity was destroyed by the addition of acetone (15–40%, v/v) but not by ethanol in concentrations up to about 25% (v/v). Ammonium sulphate (70% saturation) precipitated the enzyme in a form stable for months at −5°.

Distinction from glutathione S-aryltransferase. That the enzyme coupling iodomethane to GSH was distinct from that coupling 3,4-dichloronitrobenzene, sulphobromophthalein and some other aromatic compounds to GSH (Combes & Stakelum, 1961; Booth, Boyland & Sims, 1961; Al-Kassab, Boyland & Williams, 1963; Grover & Sims, 1964) was shown by the different proportions of these activities in liver and kidney, and by their different responses to dialysis, to heat inactivation and to ammonium sulphate fractionation, as shown in Table 2. J. Booth, E. Boyland & P. Sims (personal communication) have shown that their crude preparations of glutathione *S*-aryltransferase from liver (which are stored and survive indefinitely at −2°) have negligible activity with iodomethane as substrate.

Separation and partial purification. For the study of substrate specificity it was essential to use an enzyme preparation substantially free from the glutathione *S*-aryltransferase, and partially purified enzyme also simplified assay procedures by eliminating blanks. Since most of the work *in vivo* had been concentrated on the liver it seemed preferable to continue with a study of the enzyme separated from rat liver, which also is available in greater quantity than kidney although the latter tissue would be convenient since it does not contain glutathione *S*-aryltransferase. Purification procedures were limited by the instability of the enzyme in solutions of low ionic strength such as are necessary for adsorption of proteins on ion-exchange celluloses and calcium phosphate gels. However, a simple procedure that could be completed in 1 day was developed which gave a preparation stable for many weeks at −5° and which provided negligible

Table 2. *Distinctions between enzymes coupling iodomethane and 3,4-dichloronitrobenzene to glutathione*

All experiments were carried out with extracts of rat liver and kidney. The 3,4-dichloronitrobenzene reaction was followed spectrophotometrically (Booth *et al.* 1961) and the iodomethane reaction was assayed with the pH-stat (method *c*).

Test	With iodomethane	With 3,4-dichloronitrobenzene
1. Activity/g. of liver (arbitrary units)	100	100
2. Activity/g. of kidney (arbitrary units)	70	<5*
3. Liver/kidney activity ratio	1·4:1	>20:1*
4. Activity remaining after 5hr. dialysis against deionized water:		
(*a*) Liver extract	19%	73%
(*b*) Kidney extract	21%	—
(*c*) Extract of Chester Beatty male rat liver	19%	95%*
5. Activity remaining after heating at 46° for 8min. (liver)	41%	94%
6. Activity precipitated by $(NH_4)_2SO_4$ (60% saturation) at pH7·5 (liver)	70%	10%*

* Agrees with Booth *et al.* (1961).

assay blanks: this material was used in many studies of the enzyme characteristics. The specific activity with respect to protein was sufficient to obviate the deproteinization step in all assay methods except (*d*). Fractionation of liver extract was according to the solubility of zinc proteinates in 15% ethanolic solution: it is based on methods developed by Cohn *et al.* (1950) for plasma fractionation but apparently seldom used by enzymologists in spite of several practical advantages. The only drawback was that buffers commonly used in the range pH 6–8 were unsuitable because of their power to precipitate or form complexes with zinc salts. A mixture of hydroxylamine hydrochloride, sodium acetate and triethanolamine provided a fair buffer over the range pH 3·6–8·6 without interfering with the zinc–protein interaction. Ammonium sulphate fractionation followed the zinc sulphate fractionation: use of Sephadex G-100 gel achieved a very similar fractionation to that with ammonium sulphate but was slower and required rather careful monitoring.

The preparation of a typical batch of purified enzyme is described in the Methods section and summarized in Table 3.

The activity of a typical specimen of fraction G was 0·7 μmole/min./ml. at pH 7·0 and 25° (GSH, 1·4 mM; iodomethane, 2·0 mM).

Activity with respect to pH. Fig. 2 shows the pH–activity curve for the enzyme-catalysed reaction of GSH (1·6 mM) with iodomethane (2·05 mM). The maximum enzymic rate is observed between pH 8 and 9, above which it dropped sharply. This contrasts with the non-enzymic rate, which increases steadily to a maximum at about pH 10. It seems probable that the non-enzymic reaction involves the molecular species GS^-, which will increasingly predominate over GSH as pH is raised. The fact that the enzymic rate does not increase up to pH 10 in a parallel fashion indicates that the protein is significantly affected at high pH. The fact that change of pH influences the K'_m of glutathione (see below) means that a plot of $V_{max.}$ against pH may well be significantly different from Fig. 2, which refers to an arbitrarily chosen concentration of GSH.

Substrates. The results below indicate that both the GSH and alkyl moieties are bound to the

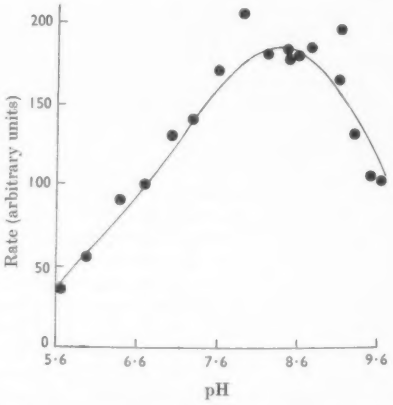


Fig. 2. Relationship of rate of reaction to pH at the chosen substrate concentration. The reaction of GSH (1·6 mM) with iodomethane (2·05 mM) was assayed for 4 min. at 25° by method *b* with purified enzyme as described in the text. Rates are corrected for non-enzymic reaction (negligible below pH 7·8).

Table 3. *Partial purification of glutathione* S-*alkyltransferase*

Separation was as described in the text. Both enzymes were assayed by method *b* (iodomethane at pH 7·0 and 25°, 3,4-dichloronitrobenzene at pH 8·0 and 37°, in order that low activity could be detected). Activities are expressed as percentages of that in fraction B, which had activities of 3·9 and 3·0 μmoles of substrate/min./g. of liver for alkyltransferase and aryltransferase respectively.

Fraction	Vol. (ml.)	Activity (% of B)	Protein (% of B)	Activity/protein ratio	Activity of GSH aryltransferase (% of B)	Alkyltransferase/aryltransferase activity ratio
A 2% NaCl homogenate*	350	(110–120	252	0·45	115	1·0)
B 2% NaCl supernatant (extract)	320	100	100	1·0	100	1·0
C Ethanol supernatant	330	100	59	1·7	—	—
D First $ZnSO_4$ supernatant	320	68	30	2·7	61	1·1
E Second $ZnSO_4$ ppt. (redissolved)	178	50	16	3·1	35	1·4
F $(NH_4)_2SO_4$ ppt.:						
(i) (48–56% saturation)	25	20	3·1	6·5	<3	approx. 7
or (ii) (47–60% saturation)	25	28	4·5	6·2	<3	approx. 10
G $(NH_4)_2SO_4$ ppt. (0–70% saturation)	—	approx. 25	—	—	<1	approx. 30

* Values derived from separate experiments.

enzyme during reaction. It is convenient to consider them separately.

(*a*) Thiol substrate. (i) Specificity. None of the following compounds reacted with iodomethane at increased rate under influence of the enzyme: L-cysteine; L-histidine; 2-mercaptopropionic acid; 2-mercaptoethylamine; 2-mercaptoethanol; 2,3-dimercaptopropan-1-ol (BAL). However, some of these compounds reacted non-enzymically with iodomethane at a greater rate and some at a lower rate than did GSH. It therefore seems impossible that the enzyme mechanism involves merely direct reaction of an 'activated iodomethane' with thiols, which themselves remain uninfluenced by the enzyme.

(ii) Kinetics. Fig. 3 shows that with iodomethane (1mM) a linear Lineweaver–Burk plot was obtained for GSH at pH7·0: K'_m (the apparent K_m at this non-saturating concentration of iodomethane) was 2·2mM. At pH6·2, K'_m was 6·0 although $V'_{max.}$ was virtually the same as that at pH7·0. This is in accord with a postulated mechanism in which the ionized form GS^- rather than GSH is bound to the enzyme. If GSH were bound $V'_{max.}$ would be expected to increase with pH while K'_m remained constant. These deductions are only valid if the comparatively small pH change has not significantly affected the protein.

(iii) Inhibitions. Besides being ineffective as a substrate, cysteine (2mM) had no influence on the reaction with GSH (2mM) as substrate (assay *e*). However, both GSSG and *S*-methylglutathione inhibited the reaction competitively (Figs. 4 and 5).

No investigation was made on the effect of varying the pH or the concentration of iodomethane on the inhibitions.

(*b*) Alkyl substrate. (i) Specificity. Potential substrates were screened by measuring disappearance of GSH when this was incubated with the substrate in the presence and absence of purified enzyme. Many were also screened with liver extract, but non-specific blanks masked the small rates of truly enzymic reaction. Table 4 shows that some compounds other than simple alkyl halides were substrates. However, not all reactive halogen compounds were substrates (e.g. iodoacetate) and the fact that the ratio of enzymic to non-enzymic rate (A/B) under fixed conditions varied widely over the range of substrates indicates that they are bound to the enzyme and that the reaction is not merely between 'activated GSH' and any alkyl compound in solution. That the alkyl substrates are actually bound to an active site is confirmed by

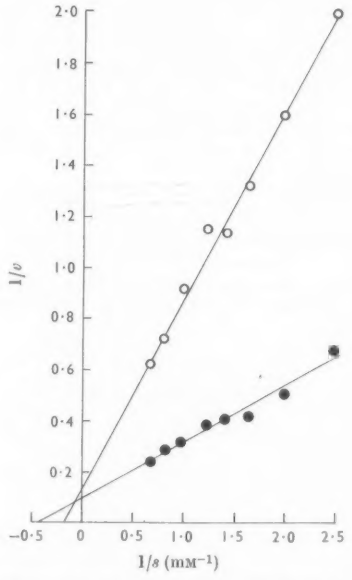


Fig. 3. Influence of pH on the Lineweaver–Burk plot for GSH. The reaction of GSH (0·4–1·5mM) with iodomethane (1mM, 200μc/m-mole) was assayed for 3min. at 25° at either pH7·0 (●) or pH6·2 (○) by method *e*. The rates are corrected for non-enzymic reaction (negligible at pH6·2).

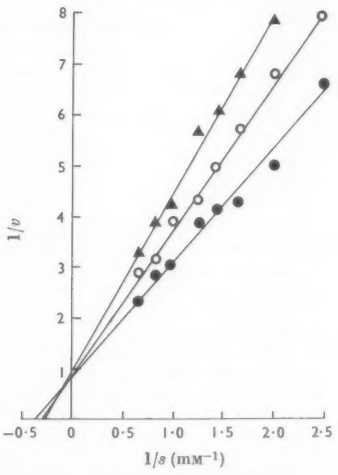


Fig. 4. Inhibition of the reaction by GSSG. Lineweaver–Burk plots are shown for the reaction of GSH (0·4–1·5mM) with iodomethane (1mM; 200μc/m-mole) in the absence of GSSG (●), in the presence of 0·8mM-GSSG (○) and in the presence of 3·2mM-GSSG (▲). The reaction was assayed for 3min. at 25° and pH7·0 by method *e*. The rates are corrected for non-enzymic reaction.

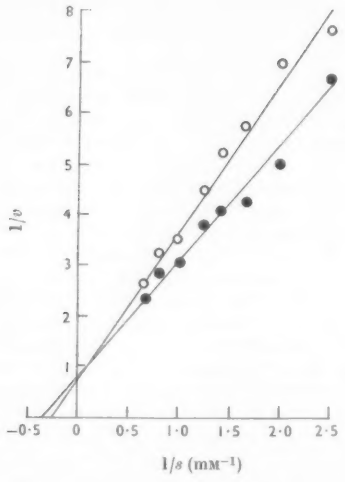


Fig. 5. Inhibition of the reaction by *S*-methylglutathione. Lineweaver–Burk plots are shown for the reaction of GSH (0·4–1·5 mM) with iodomethane (1 mM; 200 μc/m-mole) in the absence (●) and presence (○) of *S*-methylglutathione (1 mM). The reaction was assayed for 3 min. at 25° and pH 7·0 by method *e*. The rates are corrected for non-enzymic reaction.

the kinetic and inhibition experiments described below.

(ii) Kinetics. Fig. 6 shows that the rate of enzymic reaction of iodomethane and bromomethane approaches saturation in the range 0·5–3 mM, whereas the rates of the ethyl and *n*-propyl substrates increased nearly linearly with respect to concentration in this range, indicating that they had low affinity for the enzyme. A Lineweaver–Burk plot over a range of iodomethane concentrations (0·5–3 mM) shows a linear relationship (Fig. 7) with K'_m 0·37 at a GSH concentration of 2·1 mM and at pH 7·0. No determination was made to see if either the concentration of GSH or the pH influenced the result. The determination of K'_m of bromomethane was impracticable because of losses of the very volatile substrate during the assay, but these were not so great as to vitiate the observation of saturation of the enzyme by this substrate. No attempt was made to determine K'_m for the ethyl or propyl halides; values calculated from Lineweaver–Burk plots for substrates of low affinity are very inaccurate since the intercept on the abscissa is small and errors are magnified. That these substrates are bound to an active site on the enzyme is shown by the competition between them (see below).

(iii) Inhibitions. The last column of Table 4 shows the extent of inhibition that occurred when

Table 4. *Comparison of alkyl substrates and inhibitors*

The substrate survey used methods *b* and *e* at 25° and pH 7·0 with GSH (2 mM) and alkyl substrate (1·4 mM). The inhibition studies used method *e* with GSH (2 mM), inhibitor (2 mM) and iodo[^{14}C]ethane (1·8 mM). 100 rate units are equivalent to the conversion of 0·4 μmole of substrate/min. All enzymic rates refer to an arbitrarily fixed quantity of enzyme and were corrected for non-enzymic reaction.

Compound	Reaction rate (arbitrary units) Enzymic (*A*)	Non-enzymic (*B*)	Ratio *A*/*B*	Inhibition of iodoethane reaction (%)
Iodomethane	100	2	50	90
Bromomethane	40	<1	—	—
Iodoethane	20	0·4*	50	—
Bromoethane	13	<1	—	approx. 20
1-Iodopropane	13	0·16*	80	—
1-Bromopropane	6	<1	>6	5–10
2-Bromopropane	<5	<1	—	—
Bromochloromethane	<5	<1	—	—
2-Chloroethanol	<5	<1	—	None
2-Bromoethanol	<5	<1	—	None
Chloroacetaldehyde	55	55	1	20
Chloroacetic acid	<5	<1	—	None
Chloroacetamide	<5	<1	—	None
Chloroacetonitrile	20	4	5	50
Iodoacetic acid	<5	12	<0·4	—
Iodoacetamide	100	32	3	10
2-Bromopropionic acid	<5	—	—	—
3-Bromopropionic acid	<5	—	—	—
β-Propiolactone	18	3	6	50

* Extrapolated from Ingold (1953).

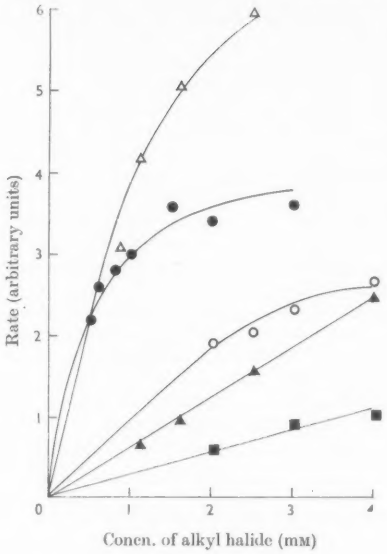


Fig. 6. Relationship of rate of enzymic reaction to alkyl halide concentration. The reaction of GSH (2–3mM) with alkyl halide (0·5–4mM; variety of specific activities) was assayed for 4–16min. at 25° and pH7·0 by method *b* (for bromomethane) or method *e*. Rates (corrected for non-enzymic reaction) are calculated in arbitrary units and are not comparable between substrates. Comparative rates are given in Table 4. ●, Iodomethane; ○, bromoethane; ▲, iodoethane; △, bromomethane; ■, 1-bromopropane.

the listed substrates (2mM) were tested as inhibitors of the reaction of iodoethane (1·8mM) with GSH (2mM). All the compounds found to inhibit the reaction were also substrates for the enzyme, and those that did not were not substrates. Characteristically the most effective inhibitors (iodomethane, propiolactone and chloroacetonitrile) were those compounds most influenced by the enzyme when they were offered as substrates (i.e. the A/B ratio in Table 4 was comparatively high for these compounds). The converse was not universally true,

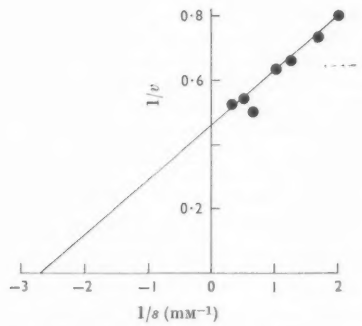


Fig. 7. Lineweaver–Burk plot for iodomethane. The reaction of GSH (2mM) with iodomethane (0·5–3mM; 10–60μc/m-mole) was assayed for 3min. at 25° and pH7·0 by method *e*. The rates are corrected for non-enzymic reaction.

Table 5. *Inhibitions of enzymic reaction of alkyl halides*

Radioactive substrates were assayed by method *e* in the presence of non-radioactive inhibitor with GSH (2mM) at pH7 and 25°. Inhibitor and substrate were added simultaneously. The reaction times were 8–16min.

Substrate	Inhibitor	Inhibition (%)
Bromoethane (2mM)	Iodoethane (3mM)	37
Iodoethane (5mM)	Iodomethane (1·9mM)	75
	Bromoethane (6mM)	40
	1-Bromopropane (8mM)	20
Iodoethane (1·8mM)	Chloroacetonitrile (3mM)	67
	β-Propiolactone (3mM)	66
	Iodoacetamide (2mM)	10
	Chloroacetaldehyde (2mM)	23
Iodoethane (1·3mM)	2-Chloroethanol (10mM)	None
	2-Bromoethanol (10mM)	None
	Chloroacetic acid (10mM)	16
	Chloroacetamide (10mM)	16
1-Bromopropane (8mM)	Iodomethane (1·9mM)	82
	Bromoethane (5·5mM)	30
	Iodoethane (5·5mM)	37

for the A/B ratio was high for the higher alkyl halides, which competed only poorly.

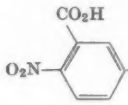
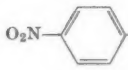
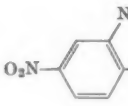
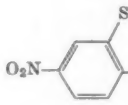
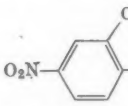
Table 5 shows the results of various inhibition experiments with pairs of alkyl halides in which the substrate was radioactive and inhibitor not. It was not possible to plot inhibition curves with iodomethane or other reactive compounds as inhibitors of more slowly reacting substrates since a considerable quantity of the inhibitor would be lost by enzymic reaction during the assay period. However, when unlabelled iodoethane was used as inhibitor for the reaction with bromo[^{14}C]ethane a linear plot was obtained of $1/v$ against inhibitor concentration according to the method of Dixon (1953) over a limited range of inhibitor concentration. Unfortunately, insufficient labelled bromoethane was available for several determinations to be made of inhibition at different substrate concentration, so that the nature of the inhibition could not be clearly demonstrated.

All the above observations suggest that the inhibitions studied were competitive. High competitive-inhibitory capacity of a compound implies that it has high affinity for the enzyme and this was found only in the smaller comparatively hydrophobic molecules: the significance of this fact in relation to the role of the enzyme is considered in the Discussion section.

Effect of sundry inhibitors and activators. *S*-Methylglutathione has been shown to inhibit the reaction (Fig. 5). It is not clear whether this is by direct competition for the GSH-binding site or by repressing the dissociation of *S*-methylglutathione from an enzyme–product complex. However, I^- ion (2mM), which is another product, also inhibits the reaction (34% with iodomethane and GSH, each 2mM). When I^- ion and *S*-methylglutathione (both 2mM), which separately inhibited the reaction 34 and 19% respectively, were both present the inhibition was 42%. Inhibition by I^- ion has not

Table 6. *Aromatic nitro compounds as inhibitors of glutathione* S-*alkyltransferase*

The reaction of iodomethane (2mM) with GSH (2mM) catalysed by the purified enzyme was assayed by the radioactive method *e*. Nitro compounds were present at 1mM in both substrate and inhibition studies. The test of reaction of nitro compounds with GSH used assay method *b*.

Compound	Inhibition (%)	Enzymic reaction with: Iodomethane	Enzymic reaction with: Glutathione
 (I)	77	*	*
 (II)	28	*	*
 (III)	96	None†	None
 (IV)	58	*	*
 (V)	31	*	approx. 3%‡

* Not tested.

† Non-enzymic action also nil during the assay period.

‡ The 3% value refers to the rate (= 100%) with iodomethane as substrate instead of (V).

been investigated over a range of substrate and inhibitor concentrations. The effect of other halide ions has not been examined except that no difference in rate was found when comparing reaction media with buffers (50mM) consisting of alkali phosphates and triethanolamine hydrochloride respectively. The back reaction of *S*-alkylglutathiones with halide ions has not been studied. Table 6 shows that a variety of aromatic nitro compounds inhibit the reaction, including 3,4-dichloronitrobenzene (V), which is a good substrate for glutathione *S*-aryltransferase (Booth *et al.* 1961). Traces of this enzyme are still present in the purified alkyltransferase (see Table 4) and this probably accounts for the observed slight reaction with GSH. However, the remoteness of the structure of these inhibitors from those of GSH and iodomethane, together with the fact that 2-amino-4-nitrophenol (compound III), which is the most effective inhibitor, will not replace either substrate in the reaction, suggests that these are probably not reversible competitors for the active site. It seems more likely that these inhibitors are bound near the active site in some way similar to the well-known binding of nitrophenols to serum albumin (Teresi & Luck, 1948).

The addition of SO_3^{2-} ion (2–8mM) to the complete reaction medium (GSH, 2mM; iodomethane, 1·2mM; pH 7·0) stimulated the enzymic reaction rate by 27%, although negligible reaction of sulphite and iodomethane occurred in the presence or absence of enzyme when GSH was omitted. Whether the reaction product was still purely *S*-methylglutathione could not be decided with the assay procedure (*e*) used, but chromatographic investigation should be informative. Since this work was completed an enzyme-catalysed reaction of sulphite with cysteine to form cysteic acid and hydrogen sulphide has been reported (Fromageot & Sentenac, 1964). The enzyme was extracted from the vitelline sac of chick embryos. It is possible that something analogous was happening in my experiments. Non-enzymic reaction was also stimulated by sulphite, possibly by increasing the degree of ionization of the thiol group; no product other than *S*-methylglutathione appears to be formed in this case.

Activity in various species. The distribution among species (Table 7) distinguishes this enzyme from the aryltransferase, the activity of which is higher in rat liver than in all other species tested (Grover & Sims, 1964): livers of the larger species had less than one-quarter of the rat-liver aryltransferase activity. The high liver alkyltransferase activities in lamb, ox, pig and monkey are not matched by high activity in the kidney of these animals.

Some observations on glutathione S-*aryltransferase of rat liver.* This enzyme has been examined in considerable detail (Booth *et al.* 1961; Al-Kassab *et al.* 1963; Grover & Sims, 1964). I have briefly examined the specificity and compared some inhibitory effects with those seen with the alkyltransferase.

Table 7. *Activity of glutathione* S-*alkyltransferase in various species*

The 2% NaCl extracts of liver and kidney were prepared as described in the text. Activity was assayed by methods *b* and *d* with GSH (1·4mM) and iodomethane (2mM) at pH 7·0 and 25°.

Species	No. tested	Activity (μmoles/min./g. of tissue)	
		Liver	Kidney
Rat (Porton strain) (♀)	>20	3–5	1·8, 2·3
Rat (Porton strain) (♂)	2	1·5, 2·1	1·5, 2·7
Mouse (A.2.G. strain) (♂)	8*	1·5	0·7
Mouse (c.52.L. strain) (♂)	8*	1·6	0·7
Guinea pig (♂)	3	1·2, 0·5, 1·8	0·2, 0·3, 0·6
Rabbit (♂)	1	5·0	0·4
Lamb	1	15·4, 14·1, 12·1	1·1, 1·5
Ox	2	3·3, 3·8	0·6 (cortex) 0·6 (medulla)
Monkey (Rhesus)	1	7·9	0·5
Pig	1	4·0	<0·1
Cat	1	3·8	0·9
Chicken	1	0·7	0·5
Toad	5*	2·2	—
Goldfish	5*	1·5	—
Human (biopsy)	1	0·9	—

* Pooled tissues

(1) Thyroxine and its precursors are the only known physiological aromatic halogen compounds in mammals, and it was therefore considered as a possible substrate. However, thyroxine (0·5mM) did not react measurably with GSH (0·5mM) when incubated for 10min. with an extract from 50mg. of liver in 12ml. at pH 8 and 37° (assay *b*). Activity as little as 6% of that towards 3,4-dichloronitrobenzene would have been detected. It seems from this experiment that the enzyme is not concerned in thyroxine metabolism.

(2) Various aromatic halogen compounds (1mM) were screened in an attempt to find a freely soluble substrate. When compared with 3,4-dichloronitrobenzene at 37° and pH 8 the total rates of reaction were: 4-iodobenzoic acid, less than 3%; 3,5-diiodoanthranilic acid, less than 3%; 2,6-dichlorophenol, 15%; 4-chloro-3,5-dimethylphenol, 15%; 4-amino-2,6-dichlorophenol, 25%; 2-amino-4-nitrophenol, less than 3%. No measure was made of non-enzymic rates. The failure of the last compound to react is significant in view of its inhibitory power towards both the alkyltransferase and aryltransferase [see (3) below].

(3) The reaction of GSH (6mM) with 3,4-dichloronitrobenzene (0·6mM) at pH 8 and 20° assayed spectrophotometrically (Booth *et al.* 1961) was inhibited by 31% by *S*-methylglutathione (6mM), by 42% by GSSG (6mM) and by 58% by 2-amino-4-nitrophenol (compound III in Table 6) (1mM). These effects are qualitatively similar to the effects on the alkyltransferase.

DISCUSSION

Identity. The ten mammalian enzymes listed in Table 8 have all been described as specifically requiring GSH as substrate. Glutathione *S*-alkyltransferase (no. 10) is clearly distinguished from three of the others (nos. 2, 7 and 8) by differences in distribution. It is distinguished from all the rest except nos. 3 and 4 (which may be identical) by its instability when dialysed against water. It is also distinguished from several by being inactivated when attempts are made to precipitate it from aqueous solution by ethanol or acetone. The only enzyme from which it cannot be clearly distinguished by these criteria is the glutathione–homo-

Table 8. *Mammalian enzymes using glutathione as substrate*

References: 1, Rall & Lehninger (1952); 2, Mills (1957); 3, Mills (1960); 4, Neubert, Rose & Lehninger (1962); 5, Neubert, Wojtczak & Lehninger (1962); 6, Racker (1955); 7, Narahara & Williams (1959); 8, Tomizawa & Halsey (1959); 9, Heppel & Hilmoe (1950); 10, Binkley & Nakamura (1948); 11, Revell & Ball (1959); 12, Goldbarg *et al.* (1960); 13, Fodor, Miller & Waelsch (1953); 14, Booth *et al.* (1961); 15, Grover & Sims (1964); 16, Boyland & Williams (1965); 17, this paper.

	Enzyme	Organs of high activity	Characteristics	References
1.	GSSG reductase	Rat liver and kidney (some activity in spleen and brain, little in blood)	(*a*) Active enzyme precipitated by acetone; (*b*) stable to water dialysis	1
2.	GSH peroxidase	Liver and erythrocytes of many species		2–5
3.	GSH–homocystine transhydrogenase	Ox liver		6
4.	GSH–oxidized CoA transhydrogenase	Ox liver	? Identical with no. 3	6
5.	GSH–insulin reductase	Rat liver	Stable to water dialysis and to 40% (v/v) ethanol	7, 8
6.	Nitroglycerine reductase	Rabbit liver, kidney stomach and uterus, and 'several organs of rat'	Pig-liver enzyme: (*a*) stable to water dialysis; (*b*) is precipitated in active form by ethanol (54–65%, v/v) at pH 5; (*c*) has optimum pH 7–8	9
7.	γ-Glutamyltransferase (glutathionase)	Predominantly kidney and pancreas of many species (also sheep brain and calf liver)		10–13
8.	GSH *S*-aryltransferase	Liver of many species Little in rat kidney	(*a*) Stable to water dialysis; (*b*) optimum pH 8	14, 15
9.	GSH *S*-epoxidetransferase	Rat liver	(*a*) Stable to water dialysis; (*b*) optimum pH 6·5	16
10.	GSH *S*-alkyltransferase	Liver and kidney of several species	(*a*) Inactivated by water dialysis; (*b*) inactivated by 30% (v/v) acetone or ethanol at 0°; (*c*) optimum pH 8–9	17

cystine transhydrogenase (no. 3), for which only limited data are available, but which is not reported to occur in kidney. It seems probable therefore that glutathione *S*-alkyltransferase is an enzyme distinct from any previously characterized as being specific for GSH.

Besides those listed above, an enzyme catalysing the production of H^+ and Br^- ions from bromochloromethane is present chiefly in liver and kidney of the rat and is precipitated from liver supernatant by ammonium sulphate between 45 and 55% saturation (Heppel & Porterfield, 1949). Although I have not been able to show bromochloromethane to be a substrate for the transferase, these characteristics match those of *S*-alkylglutathione transferase. However, the authors state that their enzyme required either GSH or cysteine and has a pH optimum of 7·1–7·4. Their Table III shows that up to 9 μmoles of Br^- ion were produced in an experiment with unfortified liver extract, which would have contained only 2 μmoles of GSH. It is obvious therefore that the major portion of the reaction was not catalysed by an enzyme utilizing GSH as substrate in stoicheiometric amount.

Function. The normal function of all the three glutathione transferases present in rat liver (Table 8) is obscure since in no case has a physiological second substrate been found. Thyroxine is almost the only organic halogen compound that has a definite place in mammalian metabolism, and this has been shown not to be a substrate for either the alkyltransferase or the aryltransferase: it is known to be excreted conjugated with glucuronic acid rather than as a mercapturic acid (Taurog, Briggs & Chaikoff, 1952). The substrates most effectively converted by the alkyltransferase are small in size and tend to be hydrophobic in character, although neither criterion is absolute (Table 4). Brodie (1956) has pointed out that hydrophobic molecules are often preferred to hydrophilic analogues as substrates for the so-called drug-metabolizing enzymes located on liver microsomes, although this is not universally true (Mazel & Henderson, 1965), and he has suggested that they exist specifically to solubilize and aid in the excretion of hydrophobic foreign compounds accidentally ingested. Whether or not a hypothesis similar to Brodie's (1956) is reasonable for the glutathione transferases, it is now becoming apparent that the production of a urinary mercapturic acid from an administered compound may depend on the existence of an appropriate glutathione transferase. However, it seems strange that a general detoxicating capacity should exist whereby aryl substrates may all be dealt with by an enzyme present only in liver, whereas alkyl substrates require one enzyme in liver and also one in kidney.

An alternative explanation of function is that a true physiological second substrate exists for glutathione *S*-alkyltransferase, although it reacts slowly compared with iodomethane. Less reactivity would be no hindrance *in vivo* where much larger amounts of enzyme are present in the liver than were used *in vitro*. Screening tests *in vivo* or with very large amounts of purified enzyme *in vitro* might reveal some hitherto unsuspected substrates that react slowly.

Problem of 2-chloroethanol. 2-Chloroethanol and 2-bromoethanol have been shown to influence the GSH concentration in the liver of rats after oral administration (Johnson, 1965), yet appeared to be unreactive in the screening tests (Table 4). It may be that these compounds are metabolized *in vivo* to compounds such as chloroacetaldehyde or ethylene oxide, which would react rapidly even without enzymic influence. A possibility that has not been excluded is that an enzyme exists in liver extracts that can catalyse the reaction of 2-chloroethanol at a rate undetected in the presence of non-specific blanks and that this enzyme was lost during the purification of the enzyme utilizing iodomethane.

I thank Dr W. N. Aldridge for much stimulating discussion and encouragement and Mrs M. A. Thomas for skilled technical assistance. Most of the work described here has been included in a Thesis for the Ph.D. award of the University of London.

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The Acidic Amino Acids of Tulip: Isolation of γ-Ethylideneglutamic acid

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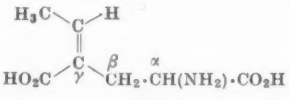
(*Received* 2 *June* 1965)

1. γ-Ethylideneglutamic acid has been isolated from fruit capsules of tulip plants. 2. The assigned structure was indicated by examining the products formed after oxidation and catalytic hydrogenation and was confirmed by nuclear-magnetic-resonance spectroscopy and by synthesis of γ-ethylglutamic acid. 3. The ability of γ-ethylideneglutamic acid to participate in transamination and decarboxylation reactions was examined.

Tulips (*Tulipa gesneriana*) are a source of several γ-substituted glutamic acids. Initially, γ-methyleneglutamic acid and γ-methyleneglutamine were obtained from tulip bulbs (Zacharius, Pollard & Steward, 1954), and identified by comparison with authentic materials previously characterized from peanut plants (Done & Fowden, 1952). Subsequently, in a survey of amino acids of liliaceous plants, Fowden & Steward (1957) concluded that tulip leaves contain smaller quantities of γ-methylglutamic acid and γ-hydroxy-γ-methylglutamic acid. A small amount of the γ-methyl derivative was isolated later (Blake & Fowden, 1964) from tulip leaves and, in better yield, from the fern *Phyllitis scolopendrium*; it was shown to be the *erythro*-L isomer. The γ-methyl and α-amino groups of γ-hydroxy-γ-methylglutamic acid isolated from the same fern possessed a similar *erythro*-L configuration.

In the survey of liliaceous plants the unidentified ninhydrin-positive compound (U144) was observed as a major constituent of tulip seeds. Chromatographic comparison showed that this compound probably was identical with an unidentified acidic amino acid isolated as a very minor component from tulip leaves (Blake & Fowden, 1964). The present account gives details of its isolation and characterization as *γ-ethylideneglutamic acid* (I) from immature fruits (capsules containing immature seeds) of tulip in which it occurs in concentrations intermediate between those of the leaf and the ripe seed.



(I)

Isolation and identification

γ-Ethylideneglutamic acid was isolated from immature tulip seed capsules by ion-exchange chromatographic techniques. The pure compound had empirical formula $C_7H_{13}NO_5$, which included a molecule of water of crystallization.

The compound could be catalytically hydrogenated and the reduction product was resolved into two spots of approximately equal intensity when paper chromatograms were developed with a 2-methylbutan-2-ol–acetic acid–water mixture, which is particularly effective for separating diastereoisomeric amino acids (Gray, Blake, Brown & Fowden, 1964).

L-Aspartic acid was the only ninhydrin-reacting product identified after oxidation of γ-ethylideneglutamic acid with acidic permanganate. The increased positive rotation observed for γ-ethylideneglutamic acid in 6N-hydrochloric acid compared with water also indicated that it possessed an L-configuration (Clough, 1918; Lutz & Jirgensons, 1930, 1931). When γ-ethylideneglutamic acid was oxidized with alkaline permanganate followed by periodate (Done & Fowden, 1952), the presence of acetaldehyde in the distillate was indicated by the piperidine–sodium nitroprusside colour reaction (Feigl, 1956). The identification of aspartate as the product of oxidation established that the unsaturated linkage (indicated by catalytic hydrogenation) could not occur in the chain before the γ-carbon atom is reached.

γ-Ethylideneglutamic acid was completely destroyed when solutions, adjusted to pH 3 with acetic acid, were autoclaved. This treatment led to a complete loss of ninhydrin-reacting material but disappearance of γ-ethylideneglutamic acid was associated with the formation, by cyclization, of a lactam (positive starch–iodide–chloroimide test; Rydon & Smith, 1952). By analogy with earlier

studies (Blake & Fowden, 1964) the lactam was presumed to be 3-ethylidenepyrrolid-2-one-5-carboxylate.

The completeness of the cyclization reaction suggested that the main carbon chain of γ-ethylideneglutamic acid contained a glutamate skeleton; a dicarboxylic amino acid containing a linear C_4 chain as in aspartate would not cyclize, and a substituted α-aminoadipic acid would yield an equilibrium mixture of open-chain and cyclized forms. In all these reactions, the behaviour of γ-ethylideneglutamic acid was akin to that of γ-methyleneglutamic acid, and as with this compound, the production of two substances after catalytic hydrogenation suggested that the unsaturated linkage must involve a carbon atom located at the branch point of the carbon skeleton. These conclusions can be rationalized only with the γ-ethylideneglutamic acid structure. The assignment of this structure was supported by the results of nuclear-magnetic-resonance spectroscopy. The spectrum, measured in deuterated water, could be integrated and indicated the correct proton number (excepting the exchangeable carboxyl and amino group protons): the presence of CH_3–CH and –CH_2–CH structures was also confirmed, and other details of the spectrum suggested that the methyl group is probably *cis*- to the γ-carboxyl group.

The γ-ethylideneglutamic acid structure was confirmed partially by an unequivocal synthesis of *γ-ethylglutamic acid* by a stepwise condensation of dibromomethane first with sodio diethyl ethylmalonate and then with sodio diethyl acetamidomalonate. The product obtained after acidic hydrolysis behaved identically with the reduction product of γ-ethylideneglutamic acid in all chromatographic solvents tested: it was separated into *threo*- and *erythro*- isomers after chromatography in 2-methylbutan-2-ol–acetic acid–water (compare above) and by high-voltage ionophoresis at pH 3·6. After racemizing the reduction product of γ-ethylideneglutamic acid by treatment with barium hydroxide, an infrared spectrum was obtained which was identical in all major details with that obtained from synthetic γ-ethylglutamic acid.

Other acidic amino acids

The acidic amino acid fraction retained by Dowex 1 was separated into more than 20 components during subsequent elution with acetic acid. In addition to γ-ethylideneglutamic acid, aspartic acid, glutamic acid, α-aminoadipic acid, γ-methylene-, γ-methyl- and γ-hydroxy-γ-methyl-glutamic acid were characterized by isolation and comparison with authentic samples. Many of the remaining compounds were unstable when treated with N-hydrochloric acid at 100° for a few hours and chromatographic identification of the hydrolysis products suggested that several were γ-glutamyl peptides, e.g. γ-glutamylvaline, γ-glutamyl-leucine and γ-glutamylphenylalanine (previously identified in the Family Liliaceae; Virtanen & Matikkala, 1961) and possibly γ-glutamylalanine, γ-glutamylserine, γ-glutamylglycine and γ-glutamylthreonine. γ-Ethylglutamic acid was not identified among the spots stable to hydrochloric acid treatment. Similarly, an attempt to identify γ-hydroxy-γ-ethylglutamic acid among this group of compounds also proved negative: substances moving close to the position that the hydroxyamino acid might be expected to occupy on two-dimensional chromatograms were treated with hydriodic acid and red phosphorus under conditions that would lead to the replacement of hydroxyl by hydrogen (Blake & Fowden, 1964), but in no case was γ-ethylglutamic acid formed.

Enzymic degradation of γ-ethylideneglutamic acid

γ-Methylene- and γ-methyl-glutamic acids were first observed to participate in transamination reactions with various keto acids by using peanut seedlings as a source of transaminases (Fowden & Done, 1953). Subsequently, a purified glutamate–aspartate transaminase from cauliflower buds was shown to utilize both C_6 acids as alternative, but relatively poor, substrates in place of glutamate (Ellis & Davies, 1961). In new experiments with enzyme preparations from peanut seedling mitochondria γ-ethylideneglutamic acid has been shown to undergo transamination with oxaloacetate at a rate comparable with that observed with γ-methyleneglutamic acid.

Glutamate decarboxylase, present in a variety of plant tissues (Fowden, 1954), is able to utilize γ-methyleneglutamic acid at about 40% of the rate of the normal substrate, glutamic acid. Now γ-ethylideneglutamic acid has been tested as a substrate for the glutamic decarboxylase of pepper fruits and tulip leaves (two of the original plant sources tested), but no decarboxylation was observed.

DISCUSSION

The identification of a further γ-substituted glutamic acid in plants possessing a range of such branched C_6 amino acids leads immediately to speculation concerning biogenetic relationships. Early experiments with peanut seedlings indicated that at least one molecule of pyruvate was incorporated into γ-methyleneglutamic acid as an intact C_3 unit (Fowden & Webb, 1958), and the possibility

has been outlined that biosynthesis involves a type of aldol condensation between two molecules of pyruvate (or some activated derivative) with the initial formation of γ-hydroxy-γ-methyl-α-oxoglutaric acid (Fowden, 1958; Marcus & Shannon, 1962). By utilizing analogous reactions, γ-ethylideneglutamic acid might result by the initial condensation of pyruvate with α-oxobutyrate although the corresponding γ-hydroxy-γ-ethylglutamate has not been detected in tulip extracts. This view would place γ-methyleneglutamic acid and γ-ethylideneglutamic acid in a similar biogenetic relationship to that existing between valine and isoleucine: an early step in the pathways leading to these two protein constituents involves the condensation of an activated acetaldehyde moiety with either pyruvate or α-oxobutyrate respectively. Unfortunately the limited distribution and low concentration of γ-ethylideneglutamic acid will make biosynthetic studies difficult even when labelled precursors are used.

Biogenetic relationships are seen also between dicarboxylic amino acids and cyclic imino acids. The conversion of glutamate into proline is the most familiar, but *erythro*-γ-hydroxy-L-glutamate is reversibly converted into 4-hydroxy-L-proline in animal tissues (Goldstone & Adams, 1963). Other pairs of compounds showing analogous structural relationships exist in plants, e.g. *erythro*-γ-methyl-L-glutamate and *trans*-4-methyl-L-proline, and γ-methylene-L-glutamic acid and 4-methylene-DL-proline (Fowden, 1964), but no imino acid comparable with γ-ethylideneglutamic acid is known in higher plants. Recently an *N*-methyl-4-propylproline moiety has been shown to be a component of an antibiotic, lincomycin, produced by *Streptomyces lincoleensis* (Hoeksema *et al.* 1964), while the 4-ethylhygric acid (4-ethyl-*N*-methyl-L-proline) was identified subsequently in another lincomycin-type antibiotic present in the same culture filtrates (Argoudelis, Fox, Mason & Eble, 1964).

EXPERIMENTAL

Chromatographic and ionophoretic techniques. Solvents used for paper chromatography were: 75% (w/w) phenol solution in the presence of NH_3; a one-phase mixture of butan-1-ol–acetic acid–water (90:10:29, by vol.); the upper phase of 2-methylbutan-2-ol (technical grade; British Drug Houses Ltd.)–acetic acid–water (10:1:10, by vol.). Amino acids were detected by spraying the chromatograms with a 0·1% solution of ninhydrin in 95% (v/v) ethanol while the starch–iodide–chloroimide technique of Rydon & Smith (1952) was used to locate the pyrrolidonecarboxylic acids (lactams) obtained after cyclization of the γ-substituted acids.

Paper-ionophoretic separations were performed in buffers at pH 2·0 (formic acid–acetic acid–water, 61:97:1842, by vol.) and pH 3·6 (formic acid–pyridine–water, 10:1:189, by vol.) by using a horizontal method based on that of Gross (1961).

Isolation of γ-ethylideneglutamic acid (I). Fruit capsules (40 kg.) were collected, deep-frozen and minced. After thawing the mush was pressed (6000 lb./in.^{2}) and the juice adjusted to pH 4·6. Protein was coagulated by warming to 60° and after stirring in absorbent charcoal the mixture was filtered. The clarified extract (30 l.) was applied to a squat column containing 20 lb. of Zeo-Karb 225 (H^+ form; mesh 52–100). All ninhydrin-positive material was held by the resin which was then thoroughly washed with 60 l. of water. The amino acids were displaced from the column by 2 N-NH_3 (35 l.) without fractionation and the eluate was concentrated by evaporation *in vacuo* at 40° to remove NH_3.

This eluate fraction (2·5 l., pH 5·8) was applied to a column (80 cm. × 5·5 cm. diam.) of Dowex 1 (X10; acetate form; mesh 100–200) to retain acidic amino acids. Neutral and basic amino acids were washed through with CO_2-free water (15 l.) before the acidic substances were displaced by gradient elution with 0·25–2 N-acetic acid. Fractions (50 ml.) were collected after the breakthrough of the first acid, glutamic acid. γ-Ethylideneglutamate (initial colour brown with ninhydrin) was present in fractions 2–14 together with an approximately equal amount of glutamic acid. Small amounts of γ-methyleneglutamic acid and γ-methylglutamic acid also were present, especially in later fractions of this range. Aspartic acid was found in low concentration in fractions 19–49 and γ-hydroxy-γ-methylglutamic acid in fractions 60–85. This γ-hydroxy-amino acid crystallized (0·12 g.) when fractions 62–82 were reduced in volume to 25 ml. Altogether 220 fractions containing ninhydrin-positive material were collected: many of the substances tentatively identified as γ-glutamyl peptides occurred in discrete groups of fractions between nos. 92 and 220.

Fractions 2–13 were combined and evaporated to dryness *in vacuo* below 50° to remove acetic acid. The residue was redissolved in 250 ml. of water and adjusted to pH 6·0 by addition of dilute NH_3. This solution was fractionated on a second column (125 cm. × 2 cm. diam.) of Dowex 1 (X10; acetate form; mesh 100–200), 0·25 N-acetic acid being used for elution. Fractions (5 ml.) were collected as glutamic acid appeared in the eluate. γ-Ethylideneglutamic acid was present in fractions 44–151; glutamic acid was the only other component of these fractions. The residue (4·6 g.) obtained after evaporating combined fractions 55–142 was redissolved in 50 ml. of hot water, and the solution deposited crystals of γ-ethylideneglutamic acid (0·44 g.) when stored overnight at 2°. Ethanol (1 vol.) was added to the mother liquor (3 vol.), when a second batch of crystals (0·58 g.) was collected. The two samples were combined and recrystallized from water (10 ml.) to yield 0·70 g. of pure γ-ethylideneglutamic acid (Found: C, 43·9; H, 6·8; N, 7·1; loss at 105°, 9·2. $C_7H_{11}NO_4,H_2O$ requires C, 44·0; H, 6·8; N, 7·3; H_2O, 9·4%). The amino acid had $[\alpha]_D^{20}$ + 21° (*c* 2·8 in water) and + 38·3° (*c* 1·4 in 6 N-HCl).

Synthesis of γ-ethyl-DL-glutamic acid. Sodium (2·4 g.) was added to dry ethanol (45 ml.) and then diethyl ethylmalonate (19 ml.) and excess of dibromomethane (20 ml.) were slowly added. The mixture was refluxed for 3 hr. and then insoluble sodium bromide was removed by filtration. Ethanol and excess of dibromomethane were removed on an

oil bath at 120°, leaving crude diethyl 2-bromomethyl-2-ethylmalonate. This intermediate was redissolved in dry ethanol (80 ml.) containing sodium (2·3 g.) and diethyl acetamidomalonate (22 g.). The mixture was now refluxed for 8 hr., precipitated sodium bromide was again removed by filtration, and most of the ethanol was distilled off. The residue was hydrolysed by refluxing with 6 N-HCl (200 ml.) for 5 hr.: crude γ-ethylglutamic acid hydrochloride remained after removal of HCl by evaporation *in vacuo*.

γ-Ethylglutamic acid was separated from other reaction products by using anion-exchange chromatography. The crude γ-ethylglutamic acid hydrochloride was redissolved in water (200 ml.) and adjusted to pH 6·0 by addition of NH_3. The solution was applied to a column (25 cm. × 5 cm. diam.) of Dowex 1 (X10; acetate form; mesh 100–200) when only γ-ethylglutamic acid was held, other ninhydrin-positive by-products being washed through with water. γ-Ethylglutamic acid was displaced with 0·5 N-acetic acid and was present in ten 50 ml. fractions. Paper-chromatographic examination of these with 2-methylbutan-2-ol–acetic acid–water as solvent showed that the first seven fractions contained both diastereoisomers (total yield, 2·8 g.) but the last three (yield 0·34 g.) contained only the slower-moving isomer; by analogy with the behaviour of the diastereoisomers of γ-methylglutamic acid (Blake & Fowden, 1964), the slower-moving isomer was probably the *erythro* form (Found for the mixed diastereoisomers: C, 47·6; H, 7·5; N, 8·2. $C_7H_{13}NO_4$ requires C, 48·0; H, 7·4; N, 8·0%).

Reduction of γ-ethylideneglutamic acid. Hydrogen was bubbled through a solution of γ-ethylideneglutamic acid (2 mg./ml.) in 2% (v/v) acetic acid in the presence of platinum oxide (5 mg./ml.). Reduction was complete in 30 min. at 20°. The reduction product gave a single spot (blue–purple) on chromatograms developed in phenol–NH_3 (R_F 0·41) and in butan-1-ol–acetic acid–water (R_{Leu} 0·72), but it was resolved into two compounds (R_{Leu} 0·71 and 0·77) when 2-methylbutan-2-ol–acetic acid–water was used as solvent. The reduction product could not be separated from synthetic γ-ethylglutamic acid when the two were co-chromatographed in each of these solvents.

The diastereoisomeric reduction product was separated by paper ionophoresis in the pH 3·6 buffer during 3 hr. at 60 v/cm.; under these conditions the slower and faster isomers in 2-methylbutan-2-ol–acetic acid–water travelled 4·6 and 2·6 cm. respectively towards the anode, and glutamic acid moved 3·9 and aspartic acid 15·6 cm. The synthetic γ-ethylglutamic acid (mixed diastereoisomers) again behaved like reduced γ-ethylideneglutamic acid. Ionophoresis at pH 1·9 failed to resolve the diastereoisomeric mixture.

Oxidation of γ-ethylideneglutamic acid. The amino acid (200 mg.) was dissolved in 5% (w/v) H_2SO_4 and maintained at 50°. A 1% $KMnO_4$ solution in 2% (w/v) H_2SO_4 was added dropwise over a period of 2–3 hr. until a pink colour persisted. Sulphate was precipitated with barium and removed with some MnO_2 by filtration. Aspartic acid was the predominant ninhydrin-positive compound in the filtrate. It was separated by adsorption on a small Dowex 1 column eluted with 0·5 N-acetic acid and obtained as a residue (39 mg.) on evaporation. It had $[\alpha]_D^{20} + 22°$ (*c* 2 in 6 N-HCl); authentic L-aspartic acid has $[\alpha]_D^{20} + 24·6°$ in 6 N-HCl.

γ-Ethylideneglutamic acid (12 mg.) was dissolved in 0·1 N-NaOH to give a solution of pH 7·0. The solution was maintained at 5° and 1% $KMnO_4$ (1 ml.) was added dropwise over 30 min. Now 0·05 g. of $HIO_4,2H_2O$ was added and the mixture stood at laboratory temperature for 1 hr. Residual periodate was precipitated by addition of $Ba(OH)_2$ and was removed by centrifugation. The supernatant was made slightly acid with H_2SO_4 and then distilled in a stream of N_2. The distillate when passed into 1 ml. of the piperidine–sodium nitroprusside reagent of Feigl (1956) gave a strong blue colour indicative of the presence of acetaldehyde. However, other aldehydes may react less sensitively with this reagent.

Cyclization of γ-ethylideneglutamic acid. A solution of the amino acid (5 mg./ml.) in acetic acid at pH 3·0 was autoclaved at 120° for 3 hr.: at the end of this time no ninhydrin-positive material remained but paper chromatography indicated the presence of an organic acid (aniline–xylose reagent) which also reacted as a lactam (starch–iodide–chloroimide test). By analogy with the behaviour of glutamic acid and γ-methyleneglutamic acid (Blake & Fowden, 1964), which cyclized slightly more slowly under these conditions, this lactam was presumably 3-ethylidene-pyrrolid-2-one-5-carboxylic acid. It had R_{Leu} 1·23 on chromatograms run in butan-1-ol–acetic acid–water (pyrrolid-2-one-5-carboxylic acid obtained from glutamic acid had R_{Leu} 0·96).

Other compounds. Tentative evidence for the presence of various γ-glutamyl peptides in fractions eluted from the Dowex 1 column was obtained by showing that the original substances were hydrolysed by N-HCl during 2–4 hr. at 100° to give glutamic acid and the appropriate neutral amino acid in the approximately equivalent amounts. The assessment of a γ-peptide link rests only upon the ease with which the compounds were hydrolysed (an α-peptide link normally splits much more slowly under the conditions described).

Infrared spectroscopy. The product obtained after catalytic hydrogenation of γ-ethylideneglutamic acid was racemized by treatment with 5 N-$Ba(OH)_2$ for 48 hr. at 100°. The i.r.-absorption spectrum of the racemized material (consisting of a mixture of approximately equal quantities of the diastereoisomeric-DL-forms) was determined in Nujol (liquid paraffin) and compared with the spectrum given by synthetic γ-ethylglutamic acid. The two spectra were identical in respect to all major absorption peaks.

Nuclear-magnetic-resonance spectroscopy. The spectra of γ-ethylideneglutamic acid and γ-methyleneglutamic acid were determined in D_2O, τ values (±0·02 p.p.m. except where stated) being obtained by reference to the methyl resonance peak of 2-methylpropan-2-ol used as internal standard. The two spectra possess many points of similarity: the α- and β-protons of both compounds give rise to the ABX type of spectrum (the two β-protons differ slightly in their respective environments) and τ_α and τ_β values are almost identical in each case. In γ-methyleneglutamic acid, the high field methylene δ-proton (τ −4·38) is markedly broadened, probably through coupling to one of the H_β, and is probably *trans*- to C_β. The coupling constant between the two δ-protons is small (1·0 cyc./sec.). In γ-ethylideneglutamic acid the methine δ-proton is not significantly broadened, indicating that the methyl group is probably *trans*- to the β-carbon atom. This spectrum shows the expected coupling ($J_{\delta\epsilon}$ = 7·6 cyc./sec.) between the methyl ε- and methine δ-protons and the low field shift of the methine δ-proton is due to the influence of the adjacent methyl group. τ values (p.p.m.) are as follows: H_α −2·70; the two

$H_\beta - 1{\cdot}59 \pm 0{\cdot}08$; $H_\delta - 4{\cdot}38$ and $-4{\cdot}82$ for γ-methyleneglutamic acid, and $H_\alpha - 2{\cdot}60$; the two $H_\beta - 1{\cdot}61 \pm 0{\cdot}08$; $H_\delta - 5{\cdot}69$ and $H_\epsilon - 0{\cdot}59$ for γ-ethylideneglutamic acid.

Transamination and decarboxylation studies. Transamination was studied with a mitochondrial preparation from 3-day-old peanut seedlings (grown at 30°), which contain the branched C_6 γ-substituted glutamic acids. The mitochondrial fraction was isolated from seedlings (100g.) by following the procedure of Blake & Fowden (1964); it was resuspended in 0·05M-potassium phosphate buffer, pH 8·0, final vol. 2·5ml., and used as a source of transaminases. Reaction mixtures, which were maintained at 30°, contained 0·03M-oxaloacetate (pH 8·0), 0·03M-amino acid (pH 8·0, glutamic acid, γ-methyleneglutamic acid or γ-ethylideneglutamic acid) and 0·3ml. of transaminase preparation in a final volume 0·5ml. The relative amounts of transamination occurring with the three amino acids after various time-intervals were estimated after chromatographic separation of the common product, aspartic acid. At 15 and 30min. considerably more transamination had occurred with glutamic acid than with either of the unsaturated acids; the latter compounds reacted at approximately the same rate. After periods of 1hr. or longer, an equal amount of aspartic acid had been formed from each of the three substrates, indicating the attainment of a similar equilibrium in each case.

Decarboxylation studies were performed with extracts of *Capsicum* fruits and tulip leaves by following the procedure of Fowden (1954). Although decarboxylation of glutamic acid proceeded to more than 90% completion and that of γ-methyleneglutamic acid to about 70% completion in 4hr. at 27°, no reaction could be detected with either γ-ethylideneglutamic acid or synthetic γ-ethylglutamic acid.

The author acknowledges with gratitude the help given by Dr M. D. Johnson, who determined and interpreted the nuclear-magnetic-resonance spectra, and by Mrs Jean Retallick, who rendered expert technical assistance.

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Biochem. J. (1966) **98**, 62

Incorporation of Injected [^{32}P]Phosphate into the Phosphoinositides of Subcellular Fractions from Young Rat Brain

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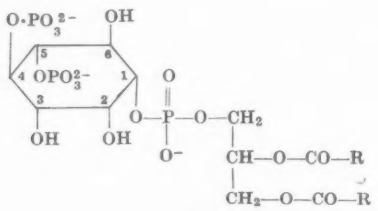
(*Received* 8 *June* 1965)

1. Six subcellular fractions were prepared from the brain of 3-week-old rats after the injection of [^{32}P]orthophosphate into the subarachnoidal space. The fractions were characterized by chemical and enzymic determinations and by electron microscopy. 2. The highest concentration of phosphoinositides was found in the microsomal fraction. 3. After an exchange period of 4hr. *in vivo*, the specific radioactivity of phosphatidylinositol was high in the nuclear, mitochondrial and supernatant fractions, and that of diphosphoinositide was high in the nuclear, microsomal and supernatant fractions. Triphosphoinositide specific activity was highest in the myelin fraction. 4. Specific activities (counts/100sec./μg. of P) were in the following order for all fractions except myelin: diphosphoinositide > phosphatidylinositol > triphosphoinositide. For myelin the order was: diphosphoinositide > triphosphoinositide > phosphatidylinositol. 5. Phosphatidylinositol extracted from a tissue fraction by acidified chloroform–methanol had roughly twice the specific activity of that extracted by neutral solvents. The same applied to diphosphoinositide.

The three inositol lipids of brain, phosphatidylinositol, diphosphoinositide and triphosphoinositide, have closely related structures. Isotope-incorporation experiments in a number of Laboratories led to the suggestion that biosynthesis of triphosphoinositide involves two successive phosphorylations, that of phosphatidylinositol to diphosphoinositide and that of diphosphoinositide to triphosphoinositide (Wagner, Lissau, Hölzl & Hörhammer, 1962; Ellis & Hawthorne, 1962; Brockerhoff & Ballou, 1962*a*,*b*). In addition, the rapid incorporation of [^{32}P]phosphate *in vivo* into the 4- and 5-phosphate groups of triphosphoinositide (I) prompted speculations about the physiological significance of this lipid, possibly in connexion with cation transport (Thompson & Dawson, 1962; Ellis & Hawthorne, 1962).



(I)

Colodzin & Kennedy (1964) have shown that a microsomal fraction from brain phosphorylates phosphatidylinositol to diphosphoinositide. A similar phosphorylation has been described with rat-liver mitochondria (Galliard & Hawthorne, 1963). Apart from these two brief reports, there is little information on the labelling of phosphoinositides in different subcellular fractions.

In the present work the labelling from inorganic [^{32}P]phosphate *in vivo* of the three phosphoinositides in subcellular fractions of rat brain is compared. Young rats were chosen, in which myelination was still in progress. The synthesis of inositol lipids is likely to be more active at this stage than in the adult (Agranoff, 1960).

EXPERIMENTAL

Subarachnoidal injections. Three-week-old rats weighing about 40g. were injected by the method of Ansell & Chojnacki (1963). The animals were anaesthetized by the intraperitoneal injection of Avertin (2·5%, w/v, in aq. 0·9% NaCl solution; Winthrop Laboratories, New York, N.Y., U.S.A.) at a dose of 0·05ml./10g. body wt. After the forehead was shaved, a 20-gauge needle fitted to an Agla micrometer syringe (Burroughs, Wellcome and Co., Beckenham, Kent) was inserted obliquely into the subarachnoid space between the frontal lobes. Occasionally, when the skulls were opened later, traces of pricking were seen on the brain surface, but the bulk of the needle tip must have been in the subarachnoid space. A volume of 30μl. of carrier-free [^{32}P]orthophosphate solution (50–160μc) was

injected in this way. Then 4hr. later the rats were killed and their brains removed, this equilibration period being sufficient to give significant labelling of di- and tri-phosphoinositide.

Subcellular fractionation. For each experiment three brains (including cerebellum and medulla oblongata) were used. The combined weight was approx. 4·2g. The fractionation was done at 4°. The brains were homogenized in 0·32M-sucrose–0·05mM-$HgCl_2$ adjusted to pH7·4 by the addition of a small quantity of solid tris, the final volume being 50ml. The $HgCl_2$ was added in the hope of inhibiting triphosphoinositide hydrolases (Thompson & Dawson, 1964; Dawson & Thompson, 1964). However, at concentrations greater than 0·05mM, $HgCl_2$ affected the fractionation process, causing partial precipitation of protein. It was successfully used in the fractionation of intestinal mucosa at a concentration of 2mM (Hübscher, Smith & Gurr, 1964). In the present work only the initial homogenization was done in the presence of $HgCl_2$.

A 10ml. portion of the homogenate was set aside and the remainder treated by the method of Whittaker (1959) and Nyman & Whittaker (1963) for the preparation of nuclear, myelin, nerve-ending ('synaptosomal'), mitochondrial, microsomal and supernatant fractions. The myelin, synaptosomal and mitochondrial fractions obtained by density-gradient centrifugation were collected as the interfacial band including the corresponding supernatant layer and were not further spun down as pellets. Nuclear and microsomal fractions were obtained as pellets and resuspended by homogenization in 0·1M-sucrose. Samples of each fraction were taken for protein determination by the biuret method of Weichselbaum (1946) and the lipids were extracted from the remainder.

To obtain some indication of the purity of the subcellular fractions several chemical and enzymic assays were made with brains from rats that had not received [^{32}P]phosphate. To overcome any inhibition by Hg^{2+} ions, L-cysteine (final concn. 2mM) was added to the enzyme assay system. Succinate dehydrogenase was measured as succinate–2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride oxidoreductase (EC 1.3.99.1) by the method of Pennington (1961). Glucose 6-phosphatase (D-glucose 6-phosphate phosphohydrolase, EC 3.1.3.9) was assayed by the method of Swanson (1955), but did not prove to be active enough to act as a marker enzyme in the brain subcellular fractions (cf. Aldridge & Johnson, 1959). Cholesterol was estimated by the method of McIlwain & Rodnight (1962), and DNA by that of Burton (1956). Dr J. B. Finean of this Department also examined the fractions by electron microscopy.

Extraction of lipids. The sucrose suspension of each subcellular fraction was first mixed with trichloroacetic acid solution (final concn. 5%, w/v). The mixture was centrifuged at 2500*g* for 15min. and as much as possible of the supernatant was then drained off. The precipitate was triturated with 20ml. of acetone and centrifuged as before. The residue was next extracted with 20ml. of ethanol, the acetone and ethanol extracts being combined. After these preliminary extractions the residue was extracted three times at 37° for 20min. with 20ml. of acidified chloroform–methanol [400ml. of chloroform–methanol (2:1, v/v) mixed with 1ml. of conc. HCl (cf. Dittmer & Dawson, 1961)]. These extracts were combined and the residual solid was discarded. The acetone–ethanol and chloroform–methanol–HCl extracts were separately treated as follows. Each was evaporated to dryness *in vacuo* and the residue dissolved in 10ml. of chloroform–methanol (2:1, v/v). This solution was shaken with 2ml. of 10mM-$CaCl_2$ and centrifuged (Folch, Lees & Sloane-Stanley, 1957). The aqueous layer was removed and replaced by a similar volume of fresh upper layer. After shaking, the mixture was centrifuged and the washed chloroform layer evaporated to dryness *in vacuo*. The residual lipid was dissolved in approx. 0·2ml. of chloroform–methanol (2:1, v/v) for application to paper chromatograms.

Paper chromatography. Formaldehyde-treated papers (Hörhammer, Wagner & Richter, 1959; Wagner, Hölzl, Lissau & Hörhammer, 1963) were prepared as follows by a slight modification of the method of Letters (1964). Fifty sheets of Whatman 3MM paper (23cm.×50cm.) were wrapped in a protective sheet of paper and soaked overnight in 2·7l. of a mixture of aq. 40% (w/v) formaldehyde and acetic acid (2:1, v/v) in a 5l. beaker covered with a glass plate. The covered beaker and its contents were then transferred to an oven at 110° for 5hr. Excess of formaldehyde vapour was removed by attaching a water pump to the vent at the top of the oven. After 5hr. the oven was

Table 1. *Components of brain subcellular fractions*

Experimental details are given in the text. The values for protein are means ± S.E.M. of seven determinations, three to four brains being combined for each determination.

	Protein (mg./g. of fresh brain)	DNA (μg. of DNA P/g. of fresh brain)	Succinate dehydrogenase (activity as % of homogenate)	Cholesterol (mg./g. of fresh brain)	Lipid inositol (μmoles/g. of fresh brain)	Lipid inositol (μmoles/100mg. of protein of fraction)
No. of rats	22	6	3	6	9	
Homogenate	161±20	127	100	12·0	1·38	0·85
Nuclear fraction	19±3	133	9·5	1·4	0·14	0·72
Myelin fraction	23±2	1·5	3·2	2·9	0·20	0·97
Nerve-ending fraction	36±2	2·6	17·0	3·6	0·36	1·05
Mitochondrial fraction	27±2	4·3	65·7	1·5	0·27	1·10
Microsomal fraction	17±1	1·1	0	2·1	0·21	1·30
Supernatant	29±1	2·6	0	0·4	0·05	0·22
Recovery (%)	94	114	95	99	94	

turned off and allowed to cool overnight. The papers were then washed for 8 hr. in running tap water followed by five washes in deionized water. They were then allowed to stand overnight in deionized water and were finally hung up to dry at room temperature. As much as 100 μg. of lipid P could be applied to these papers in a band 2·5 cm. wide.

The papers were chromatographed at room temperature by the descending technique with the upper phase of butan-1-ol–acetic acid–water (4:1:5, by vol.) equilibrated for 2 days with 0·125 vol. of diethyl ether (Letters, 1964); this produced two layers, of which the upper was used. Chromatography took 12–16 hr., after which the papers were dried and radioautographs prepared by superimposing them on X-ray film in cassettes for 3–5 days. The film was then developed and fixed in the usual way. For non-radioactive spots or confirmation of the presence of lipids, the chromatograms were soaked for 30 min. in a solution (0·002%, w/v) of Nile Blue in 0·1N-H_2SO_4 and washed for 1 min. in running tap water. Spots containing 1 μg. of phospholipid P could be detected by this method. The identity of the spots corresponding to phosphatidylinositol, diphosphoinositide and triphosphoinositide was confirmed by determination of P/inositol ratios. Values of 0·78, 1·8 and 3·2 respectively were obtained. For these determinations the areas of the chromatograms containing the phospholipids were cut out. Paper blanks of the same size were cut from parts of the chromatogram between the inositide spots.

Phosphate estimation. Control areas and lipid-containing areas from the chromatograms were cut into small pieces and digested with 1·4 ml. of 72% (w/v) $HClO_4$, together with 0·02 ml. of 1% (w/v) ammonium molybdate as catalyst. The colourless digest was then treated by the method of Bartlett (1959) for the determination of phosphate. The accurate measurement of 0·5 μg. of P was possible by this method.

Measurement of radioactivity. The samples from the phosphate determination were counted in a 10 ml. liquid Geiger–Müller tube with 6% efficiency. Since there were considerable variations in specific activity between experiments, results were always related to the specific activity of homogenate phosphatidylinositol (see Table 3).

Inositol estimation. Samples were dissolved in 2–3 ml. of 6N-HCl and hydrolysed in sealed tubes at 110° for 24 hr. Inositol was estimated in the hydrolysate by the microbiological method of Norris & Darbre (1956).

RESULTS

Electron microscopy of the subcellular fractions indicated some contamination of the nuclear fraction with myelin, but the remaining fractions were relatively homogeneous. Table 1 gives further information on the composition of the fractions. Mitochondrial and nerve-ending fractions contained the bulk of the succinate dehydrogenase, and almost all the DNA was in the nuclear fraction. All the membrane fractions contained lipid-bound inositol, but the quantity per mg. of protein was lowest in the nuclear fraction and highest in the microsomal fraction.

Table 2 gives the distribution of phosphatidyl-

Table 2. *Distribution of phosphoinositides among brain subcellular fractions*

Experimental details are given in the text. The first column of values for each compound shows μmoles or mμmoles of phosphoinositide/100 mg. of protein expressed as means ± S.E.M. with the numbers of experiments in parentheses, three brains being used for each experiment. The second column for each compound gives the percentage recovery in each subcellular fraction, that of the homogenate being taken as 100%. The third column gives the relative enrichment, obtained by dividing μmoles of phosphoinositide/100 mg. of protein in the fraction by the corresponding value for the homogenate.

	Phosphatidylinositol			Diphosphoinositide			Triphosphoinositide		
	(μmoles/100 mg. of protein)	(% recovery)	(enrichment)	(mμmoles/100 mg. of protein)	(% recovery)	(enrichment)	(mμmoles/100 mg. of protein)	(% recovery)	(enrichment)
Homogenate	1·11 ± 0·12 (5)	100	1·00	109 ± 16 (5)	100	1·00	27 ± 4 (5)	100	1·00
Nuclear fraction	0·85 ± 0·06 (5)	8	0·76	35 ± 6 (5)	3	0·32	approx. 10* (5)	6	0·38
Myelin fraction	1·20 ± 0·16 (5)	13	1·08	69 ± 17 (5)	8	0·63	25 ± 13 (5)	12	0·92
Nerve-ending fraction	1·50 ± 0·20 (5)	28	1·35	125 ± 25 (5)	23	1·14	24 ± 6 (5)	17	0·88
Mitochondrial fraction	1·34 ± 0·01 (5)	21	1·21	133 ± 16 (5)	21	1·22	22 ± 3 (5)	13	0·80
Microsomal fraction	1·75 ± 0·17 (5)	16	1·58	137 ± 24 (5)	13	1·25	43 ± 10 (5)	15	1·53
Supernatant	0·21 ± 0·01 (5)	4	0·19	12 ± 2 (4)	2	0·11	Trace (5)	—	—
Recovery of P (%)	92			71			63		
Recovery of radioactivity (%)	95			85			69		

* Values ranged from 0 to 30.

Table 3. *Relative specific activities of the phosphoinositides in brain subcellular fractions*

Experimental details are given in the text. The values are given relative to the specific activity of homogenate phosphatidylinositol and are expressed as means ±S.E.M., with the numbers of experiments in parentheses, three brains being used for each experiment. The actual specific activities of phosphatidylinositol in the homogenate varied from 190 to 1720 counts/100 sec./μg. of P. The dose of [^{32}P]phosphate injected into each rat varied between 50 and 160 μc.

	Phosphatidylinositol	Diphosphoinositide	Triphosphoinositide
Homogenate	100 (5)	135±35 (5)	93±22 (5)
Nuclear fraction	134±6 (5)	228±41 (5)	approx. 26 (2)
Myelin fraction	59±6 (5)	150±27 (5)	141±46 (4)
Nerve-ending fraction	79±7 (5)	139±32 (5)	65±8 (5)
Mitochondrial fraction	131±9 (5)	141±39 (5)	61±11 (5)
Microsomal fraction	106±9 (5)	177±45 (5)	80±26 (5)
Supernatant	144±9 (5)	approx. 261 (3)	—

inositol, diphosphoinositide and triphosphoinositide among the various subcellular fractions. Results are also calculated to show the relative enrichment in a particular fraction when compared with the brain homogenate. For all three phosphoinositides the microsomal fraction showed the greatest enrichment. The nerve-ending and mitochondrial fractions also showed significant enrichment with respect to phosphatidylinositol. Microsomal, mitochondrial and nerve-ending fractions contained relatively more diphosphoinositide than the homogenate, whereas only the microsomal fraction showed enrichment of triphosphoinositide. As shown in Table 2, recoveries of both phosphate and radioactivity were poor with diphosphoinositide and triphosphoinositide.

The relatively large standard errors in Table 2 are probably due to biological variation. Myelination is still taking place in 3-week-old rats, and inositol lipid concentrations may well depend on the extent of myelination and thus vary from animal to animal. Among the particulate fractions only the mitochondrial fraction shows relatively constant phosphatidylinositol/protein ratios. This is probably because lipid and protein synthesis are closely integrated in the mitochondrion. It may also reflect the absence from mitochondria of enzymes hydrolysing phosphatidylinositol.

The specific activities of the various phosphoinositides (Table 3) are given as percentages of that of the homogenate phosphatidylinositol for comparison. Values correspond to the total inositide extracted by the solvents used (cf. Table 4). There was considerable variation among the subcellular fractions. Specific activities of diphosphoinositide and triphosphoinositide represent averages of all the phosphates in each molecule. They were usually in the sequence: diphosphoinositide > phosphatidylinositol > triphosphoinositide. The specific activity of phosphatidylinositol was high in supernatant, nuclear and mitochondrial fractions. It was relatively low (59% of homogenate) in the myelin fraction.

Table 4. *Comparison of specific activities of the phosphoinositides extracted by acidified chloroform–methanol and those extracted by acetone followed by ethanol*

Experimental details are given in the text. Values represent the specific activity of the phosphoinositide extracted by acidified chloroform–methanol divided by the specific activity of the phosphoinositide extracted by acetone and ethanol, and are expressed as means±S.E.M. of five experiments.

	Specific activity ratio	
	Phosphatidyl-inositol	Diphospho-inositide
Homogenate	2·0±0·13	2·3±0·06
Nuclear fraction	2·3±0·19	approx. 2·7
Myelin fraction	1·7±0·11	1·6±0·09
Nerve-ending fraction	1·7±0·06	1·3±0·04
Mitochondrial fraction	1·6±0·10	0·8±0·01
Microsomal fraction	2·3±0·15	3·1±0·50
Supernatant	2·0±0·07	—

The specific activity of diphosphoinositide was higher in most of the cell fractions than in the homogenate. Nuclear, microsomal and supernatant fractions appeared to be most active. For triphosphoinositide, only the myelin fraction showed a higher specific activity than the homogenate.

Though most of the phosphoinositides appeared in the acidified chloroform–methanol extract, small amounts were found in the combined acetone and ethanol extracts. In all cell fractions except mitochondria the less firmly bound phosphatidylinositol and diphosphoinositide (acetone–ethanol extract) had consistently lower specific activities, approximately half those of the corresponding lipids in the acidified chloroform–methanol extract.

Table 4 summarizes the results. The differences were most marked in the microsomal and nuclear fractions.

DISCUSSION

From the protein and phosphatidylinositol values given in Tables 1 and 2, the content of phosphatidylinositol P/g. of fresh rat brain was 55·3μg. This was for 3-week-old rats. The corresponding value for adult guinea-pig forebrains was calculated from the results of Eichberg, Whittaker & Dawson (1964), who used a different analytical method, as 56·8μg./g. Higher values were obtained by Wagner *et al.* (1963) and Margolis & Heller (1965) for adult rat brain (93μg./g. and 108μg./g. respectively).

The greatest enrichment value for phosphatidylinositol was given by the microsomal fraction. A similar conclusion for adult guinea-pig forebrain was reached by Eichberg *et al.* (1964). Recoveries of lipid inositol (Table 1) by the microbiological assay were about 70% of the inositol values calculated from the phosphoinositide contents in Table 2. The reason for this is not known.

Hörhammer, Wagner & Hölzl (1960) reported the absence of polyphosphoinositide from the grey matter of ox brain, though small quantities are found in the grey matter of adult cat brain (P. Kemp & M. Kai, unpublished work). Eichberg & Dawson (1964) found most of the polyphosphoinositide of adult guinea-pig brain in the myelin-rich fractions. In the present experiments with 3-week-old rats, polyphosphoinositides were found in mitochondrial, microsomal and nerve-ending fractions, as well as in the myelin fraction.

Post-mortem enzymic breakdown of triphosphoinositide and diphosphoinositide is known to occur (Eichberg & Dawson, 1964). The addition of mercuric chloride to the sucrose solution used in our initial homogenization does not seem to have prevented this breakdown. A value of 15·1μg. of polyphosphoinositide P/g. of fresh brain can be calculated from Table 2, whereas the value for adult rat brain fixed in liquid air was 49·1μg. of P/g. (Eichberg & Dawson, 1964). This loss seems unavoidable in subcellular fractionation studies, and will mean that the values for triphosphoinositide in Table 2 are low whereas those for diphosphoinositide may be high. Phosphatidylinositol values are unlikely to be affected by the relatively small amounts of polyphosphoinositide involved.

Specific activities expressed as counts/100sec./μg. of P for homogenate were in the order: diphosphoinositide > phosphatidylinositol > triphosphoinositide. Wagner and his colleagues (Wagner *et al.* 1962; Hölzl & Wagner, 1964) performed similar experiments with adult rats, except that the [^{32}P]phosphate was injected subcutaneously and the brains were frozen quickly in liquid air after death. With a 4hr. equilibration they obtained the sequence: triphosphoinositide > diphosphoinositide > phosphatidylinositol. This sequence was also obtained at 6hr. after the subarachnoidal injection of [^{32}P]-orthophosphate into rabbits (Ellis & Hawthorne, 1962). The difference may be due to the fact that young rats, in which myelination was still in progress, were used in the present experiments. The specific activity of triphosphoinositide may also be affected by the enzymic breakdown referred to above. It is not easy to decide whether this breakdown would lower or raise the specific activity. If the newly synthesized triphosphoinositide is more readily hydrolysed, a lowered specific activity would be expected. It is equally difficult to predict what effect the hydrolysis would have on diphosphoinositide specific activity. An appreciable rise seems unlikely. Brockerhoff & Ballou (1962*a*,*b*) and Rossiter, McMurray & Palmer (1964), using rabbit- and rat-brain slices respectively, expressed their results as molar radioactivities and obtained the sequence: triphosphoinositide > diphosphoinositide > phosphatidylinositol. If the results of Brockerhoff & Ballou (1962*a*,*b*) are calculated as counts/100sec./μg. of P, the sequence obtained, as in the present work, is: diphosphoinositide > phosphatidylinositol > triphosphoinositide.

An unexpected finding in the present study was the high specific activity of phosphatidylinositol and diphosphoinositide in the nuclear and supernatant fractions. The most highly labelled triphosphoinositide, on the other hand, was found in the myelin fraction. Phosphatidylinositol specific activity was lowest in this fraction. Preliminary experiments with 1hr. and 2hr. equilibration periods gave very little labelling of the phosphoinositides.

Phosphatidylinositol extracted by acidified chloroform–methanol has about twice the specific activity of the same lipid in the acetone and ethanol extracts. The same applies to diphosphoinositide. These results suggest that the metabolically active phosphoinositides are bound more firmly to protein.

Thanks are due to the Medical Research Council for financial support and to the Wellcome Trust for a travel grant to M. K. We are also grateful to Mrs D. Pow, who made the biological assays of inositol, and to Dr G. B. Ansell for advice on subarachnoidal injections.

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Isolation of certain Oligonucleotides Obtained by Degradation of Deoxyribonucleic Acid

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(*Received* 19 *May* 1965)

1. Pyrimidine oligodeoxynucleotides containing one thymine nucleotide residue and up to five cytosine nucleotide residues were isolated by chromatography on DEAE-Sephadex after degrading calf-thymus DNA with diphenylamine in aqueous formic acid.

In order to test a method for the selective degradation of thymidine in oligonucleotides (Burton & Riley, 1966), it was decided to isolate the series of oligodeoxynucleotides $(C_nT)p_{n+2}$* containing one thymine nucleotide residue and different numbers of deoxycytidine nucleotide residues. This short paper describes their isolation from calf-thymus DNA by an extension of chromatographic separations on DEAE-Sephadex that were used to isolate C_np_{n+1} oligonucleotides (Petersen & Burton, 1964). The amounts of the $(C_nT)p_{n+2}$ nucleotides recovered agree closely with those expected from analyses by other methods (Petersen, 1963).

Table 1. *Isolation of* $(C_nT)p_{n+2}$ *oligonucleotides*

Calf-thymus DNA was degraded with 2% (w/v) diphenylamine in 66% (v/v) formic acid and adsorbed on to a column (28cm. × 2·3cm. diam.) of DEAE-Sephadex. P_i and cytosine nucleotides C_np_{n+1} were eluted by 2·5N-formic acid. The following fractions (200ml.) were eluted with a 0·0–0·5M linear gradient of ammonium formate in 3l. of 2·5N-formic acid. E_{280} and E_{260} values were measured after fivefold dilution with 2N-HCl.

Fraction	E_{280}	E_{280}/E_{260}	Provisional identity
1	0·127	0·68	—
2	0·119	0·75	—
3	0·900	1·41	? Mainly $(C_2T)p_4$
4	8·33	1·29	Mainly $(CT)p_3$
5	7·40	0·85	$Tp_2+(CT)p_3$
6	1·82	1·09	? $Tp_2+(C_nT_2)p_{n+2}$
7	3·15	1·19	$(C_nT_2)p_{n+2}$
8	3·82	1·01	? Mainly $(C_2T)p_4$
9	2·95	0·84	T_2p_3+other components

* Abbreviations: Since all the compounds described in this paper are deoxyribonucleotides, the prefix 'd' is omitted. C and T represent deoxycytidine and thymidine respectively; p represents phosphate esterified with the nucleosides. When p is to the right of the nucleoside symbol it is esterified with the 3′-hydroxyl of the nucleoside; when it is to the left it is a 5′-ester. Thus CpT represents deoxycytidylyl-(3′-5′)-thymidine. Sequences that are unknown or expected to consist of mixtures of isomeric nucleotides in unknown proportions are written with the nucleoside symbols in parentheses, e.g. (CT)p represents a mixture of CpT and TpC.

EXPERIMENTAL

Calf-thymus DNA, prepared by the method of Hurst (1958), was degraded with diphenylamine and formic acid and the reagents were removed by ether extraction and

Table 2. *Extinction ratios and amounts of* $(C_nT)p_{n+2}$ *oligonucleotides isolated from calf-thymus DNA*

Fractions 1–5 (Table 1) were pooled and the material was chromatographed on a column (32cm. × 1cm. diam.) of DEAE-Sephadex (HCO_3^- form). The eluting solvent was NH_4HCO_3, pH 8·8, with a 0–0·41M linear gradient in 570ml. followed by 0·41–1·1M in the next 700ml. E_{280}/E_{260} ratios were measured in 0·1N-HCl on the main portion of each peak. The amounts shown are the totals recovered and include allowances for the small amounts in the regions between the peaks. They are compared with those reported by Petersen (1963), who separated the nucleotides on paper after treatment with phosphomonoesterase.

Peak	E_{280}/E_{260}	Amount recovered (moles of pyrimidine/100moles of DNA phosphate)	Amounts reported by Petersen (1963)
$(CT)p_3$	1·26	5·15	5·19
$(C_2T)p_4$	1·54	2·70	2·64
$(C_3T)p_5$	1·65	1·19	1·39
$(C_4T)p_6$	1·65	0·50	0·39
? $(C_5T)p_7$	—	0·1	—

evaporation to dryness (Burton & Petersen, 1960; Petersen & Burton, 1964). An amount containing 2·17 μmoles of total phosphate was adsorbed on to a column (28 cm. × 2·3 cm. diam.) of DEAE-Sephadex A-25 (formate form). P_i was eluted by 2·5 N-formic acid, appearing at about 0·4 l. The cytosine nucleotides C_np_{n+1} were eluted later as a composite peak at about 1·5 l. When 3·4 l. had been collected, elution was continued with a gradient of ammonium formate in 2·5 N-formic acid (Table 1). Under these conditions, $(C_nT)p_{n+2}$ compounds are eluted first and are followed by the group of compounds $(C_nT_2)p_{n+3}$. Fractions 1–5, comprising the $(C_nT)p_{n+2}$ compounds, were pooled, freeze-dried and separated into individual components pTp, (CT)p_3, $(C_2T)p_4$ etc. by chromatography on DEAE-Sephadex (HCO_3^- form) with NH_4HCO_3 as the eluting solvent (Table 2). The amounts eluted agree well with those found by Petersen (1963) using other methods. For use in the experiments described in the following paper (Burton & Riley, 1966), the bulk of each peak, excluding the 'head' and 'tail' fractions, was pooled and the salt was removed by rotary evaporation.

For similar separations of the C_np_{n+1} nucleotides, Petersen & Burton (1964) added urea (7 M) to the eluting solvents as a precaution against losses in chromatography due to secondary binding forces. However, it now appears that the urea is not necessary because it does not improve the recovery of these pyrimidine nucleotides from columns of DEAE-Sephadex. For example, the amounts of C_np_{n+1} nucleotides recovered without the use of urea are (as moles of cytosine relative to those in C_2p_3): Cp_2, 2·05; C_3p_4, 0·41; C_4p_5, 0·20; C_5p_6, 0·05; these agree well with the highest recoveries previously obtained in the presence of urea.

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Biochem. J. (1966) **98**, 70

Selective Degradation of Thymidine and Thymine Deoxynucleotides

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(*Received* 19 *May* 1965)

1. Osmium tetroxide in dilute ammonia oxidizes various pyrimidine nucleosides at different rates. Thymidine is oxidized about 45 times as fast as deoxycytidine. The phosphate groups may be eliminated from oxidized thymine nucleotides by successive treatments with alkali and then with diphenylamine in aqueous formic acid. The reactions can be applied to the selective degradation of thymidine in oligodeoxynucleotides.

In this paper, we describe a procedure whereby thymidine may be chemically degraded with almost quantitative removal of the thymine and release of free deoxyribose. The same conditions release only a small proportion of the sugar groups from deoxycytidine. The method was devised because of its potential value in studying sequences of deoxynucleotides.

The procedure involves treatment with osmium tetroxide followed by successive treatments with dilute alkali and acid at mild temperatures. The reactions resemble those that have been reported to occur with brominated pyrimidines and their derivatives (Baudisch & Davidson, 1925; Cohn, 1956; Roberts & Friedkin, 1958). These involve conversion into a diol followed by opening of the pyrimidine ring to form an acid-labile ureidodeoxyriboside, e.g. as shown in Scheme 1. Osmium tetroxide is expected to convert thymidine into a 4,5-*cis*-diol, which should then undergo the same reactions in alkali and acid. These reactions should be the same for both ribo- and deoxyribo-nucleosides and for nucleotides. In this work we have principally studied the deoxyribose compounds because the treatment with alkali would prevent the application of the complete series of reactions to the study of nucleotide sequences in ribonucleic acids. The selectivity of the procedure that is described is due to the initial attack by the osmium tetroxide.

MATERIALS

Deoxynucleosides and deoxynucleotides. These were obtained from the California Corp. for Biochemical Research (Los Angeles, Calif., U.S.A.). Solutions of these and other nucleosides and nucleotides were standardized spectrophotometrically with the aid of values for their molar extinction coefficients as given by Burton (1959) and Burton & Petersen (1960).

Deoxyribonucleic acid. DNA was isolated from herring testes (Emanuel & Chaikoff, 1953).

Osmium tetroxide. The solid was obtained from Harrington Brothers Ltd. (London, E.C. 1) and an aqueous solution (approx. 5%, w/v) was prepared, with precautions being taken to keep the toxic vapour away from the eyes (Pease, 1960). The solution was stored at 4° and standardized spectrophotometrically. The molar extinction coefficient, $\epsilon_{260} = 2500$, was determined on a solution that had, in turn, been standardized iodometrically (Klobbie, 1898; Ayres & Wells, 1950). This coefficient is 13% higher than that found by Langseth & Qviller (1934).

Oligonucleotides (C_nT)p_{n+2}. These oligonucleotides† were isolated from calf-thymus DNA as described in the preceding paper (Burton, 1966).

Amberlite CG-50. This resin was supplied by British Drug Houses Ltd. (Poole, Dorset) and the granule size was 100–200 mesh. Fines were removed by repeated decantation from water and the resin was washed as a column with successive 2 M solutions of KOH, NaCl, HCl, KOH, NaCl and HCl. The column was washed with water until the effluent was free from chloride, converted into the NH_4^+ form with 2 N-NH_3 and washed with water (adjusted to pH 7 with aq. NH_3) until the pH of the effluent reached 7·5–8·0. This resin was stored in the refrigerator.

† Abbreviations: Only deoxynucleotides are represented by abbreviated formulae and the prefix 'd' is omitted. Pu, C and T represent a purine deoxynucleoside, deoxycytidine and thymidine respectively. The symbol p to the right of a nucleoside symbol represents a phosphate group esterified to the 3′-hydroxyl of the nucleoside; when it is to the left it is a 5′-ester. Thus CpT represents deoxycytidyl-(3′-5′)-thymidine. Sequences that are expected to consist of mixtures of isomeric nucleotides in unknown proportions are written with the nucleoside symbols in parentheses, e.g. (CT)p represents a mixture of CpT and TpC. The numbering system used for the pyrimidine ring is that in which the ethylenic bond is numbered 4–5 and the glycoside N is numbered 3 (see Cohn & Doherty, 1956).

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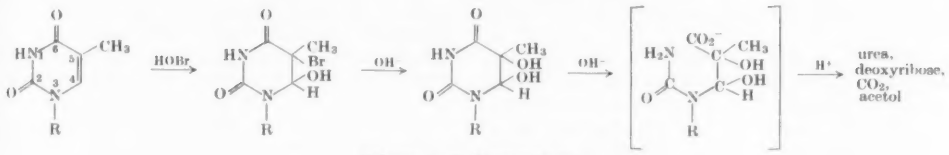

Scheme 1. R, Deoxyribosyl.

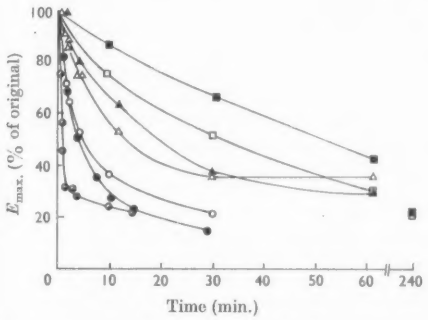

Fig. 1. Reaction of OsO_4 with pyrimidine nucleosides. Each nucleoside (2mM) was treated with 7mM-OsO_4 in 0·4N-NH_3 at 0°. Samples were removed at various times into 50vol. of ice-cold ether, excess of OsO_4 was removed by extraction into ether and the aqueous phase was diluted with 25vol. of 0·1N-HCl. Zero-time samples were taken before the addition of the OsO_4. The extinction coefficient of each sample was measured at the wavelength of maximum absorption for each nucleoside. □, Cytidine (280mμ); ■, deoxycytidine (280mμ); ○, 5-methyldeoxycytidine (286mμ); △, uridine (262mμ); ▲, deoxyuridine (262mμ); ●, 5-bromodeoxyuridine (279mμ); ◕, thymidine (267mμ).

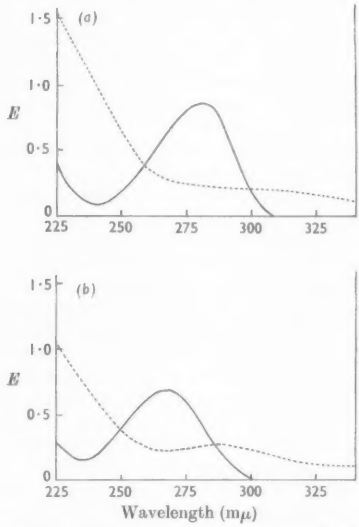

Fig. 2. Absorption spectra of nucleosides treated with OsO_4. Each nucleoside (1mM) was treated with OsO_4 in NH_3 as described for Fig. 1, and the spectra were measured after dilution in 0·1N-HCl. (*a*) Deoxycytidine; (*b*) thymidine. Solid lines are spectra before the addition of OsO_4, broken lines after the reaction was complete, i.e. 3hr. for (*a*) and 15min. for (*b*).

METHODS

Removal of osmium tetroxide. Excess of OsO_4 was removed from reaction mixtures and the oxidation stopped by extraction with diethyl ether, the partition coefficient into the ether being approx. 5. Small volumes (less than 0·5ml.) were conveniently extracted by shaking in a glass-stoppered tube with 5–10ml. of ice-cold ether, centrifuging and freezing the aqueous layer at the bottom of the tube by the use of either liquid air or solid CO_2 and ethanol. The organic solvent was then poured off, the aqueous phase thawed and the extraction repeated.

Removal of potassium hydroxide. When the products were to be examined by chromatography or electrophoresis on paper, K^+ ions were removed by treatment with Amberlite CG-50 resin (NH_4^+ form). A 5cm. × 0·7cm. diam. column was sufficient for treating reaction mixtures of up to 10ml. Nucleosides, nucleotides and their oxidized derivatives could be recovered quantitatively by washing the column with 4 bed-volumes of water that had been adjusted to pH7 with aq. NH_3 and the eluate contained less than 0·1mM-K^+ ion. Excess of NH_3 was removed by drying under vacuum in a rotary evaporator or by freeze-drying.

Phosphate estimations. P_i was estimated by the method of Chen, Toribara & Warner (1956) except in experiments where the reduced product of OsO_4 interfered. This material did not interfere in the estimation of total phosphate because it was reoxidized and volatilized by the ashing in $HClO_4$–H_2SO_4 (Hanes & Isherwood, 1949). Hence organic and inorganic phosphate could be determined separately by extraction of P_i as the phosphomolybdate complex (Berenblum & Chain, 1938, or Nielsen & Lehninger, 1955) and by ashing samples from the two phases.

Colour reaction for deoxyribose. The periodate–thiobarbituric acid reaction (Waravdekar & Saslaw, 1959) was used. In this method, free deoxyribose reacts, but combined forms such as nucleosides or nucleotides do not.

RESULTS

Effects of osmium tetroxide on the absorption spectra of pyrimidine deoxynucleosides. Osmium tetroxide at pH 9 or in dilute ammonia solution was found to lower the ultraviolet absorption of various pyrimidine deoxynucleosides at very different rates (Fig. 1) in the order: thymidine, 5-methyldeoxycytidine, deoxyuridine, deoxycytidine. Ribonucleosides react more slowly than the corresponding deoxynucleosides. The changes of spectra after prolonged reaction with osmium tetroxide are shown for thymidylate and deoxycytidylate in Fig. 2. After shorter reaction times, the spectra between 260 and 300mμ gave no indication of intermediate products that absorbed in this region. One mole of osmium tetroxide is sufficient to depress the absorption of 1 mole of thymidine to its final value at 267mμ (Fig. 3).

The rate of reaction is only slightly affected by phosphate substituents on the sugar, the relative rates being approximately: thymidine, 1·0; thymidine 5′-phosphate, 0·85; thymidine 3′,5′-diphosphate, 0·95 (Fig. 4). The linearity of these plots indicates that the reaction is first-order with respect to the thymidine compound. The rate of reaction increases in proportion to the concentration of osmium tetroxide, but the increase with the concentration of ammonia is not strictly proportional (Table 1). Essentially similar results were obtained for deoxycytidine 5′-phosphate (Table 1), and no conditions were found to alter significantly the relative rates of reaction of thymidylate and deoxycytidylate.

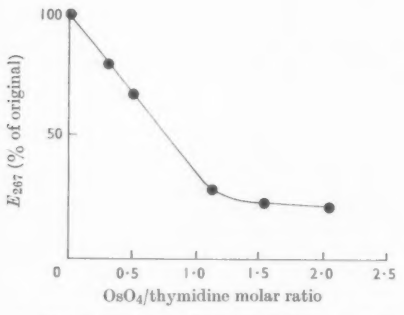

Fig. 3. Stoicheiometry of the reaction between OsO_4 and thymidine. Thymidine (2mM) in 0·1 N-NH_3 was mixed with 17mM-OsO_4 in various proportions and the mixtures were left at room temperature in glass-stoppered tubes. After 22 and after 45hr., the extinction coefficient of the nucleoside (E_{267}) was measured as described in Fig. 2. There were no significant differences between the values for the two times, showing that the reaction was complete.

Absence of appreciable action of osmium tetroxide on purine deoxynucleotides. Deoxyadenosine 5′-phosphate and deoxyguanosine 5′-phosphate (2mM) were each treated with 7mM-osmium tetroxide in 0·1 N-ammonia at 0° for 1 week and about 80% of each purine nucleotide was recovered unchanged after paper chromatography in propan-2-ol–water (7:3, v/v).

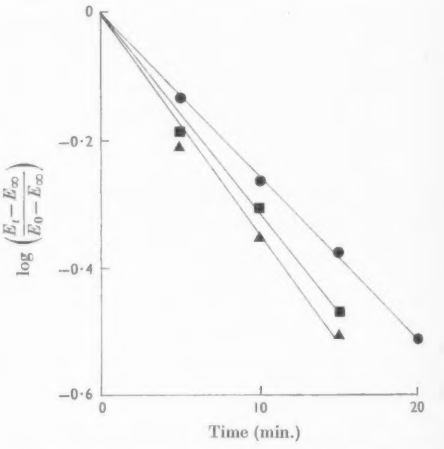

Fig. 4. Rates of reaction of OsO_4 with thymidine and thymidine phosphates. Each compound (1 mM) was treated with 3·5mM-OsO_4 in 0·1 N-NH_3 at 0°. At various times, samples were removed, excess of OsO_4 was removed and the extinction coefficient (E) was measured at 267mμ. The ordinate indicates $\log[(E_t-E_\infty)/(E_0-E_\infty)]$ where E_0 is the value of E at zero time, E_t is that at time t and E_∞ is that when the reaction was complete. ▲, Thymidine; ●, thymidine 5′-phosphate; ■, thymidine 3′,5′-diphosphate.

Table 1. *Rates of oxidation of nucleotides by ammoniacal osmium tetroxide at different concentrations of reactants*

Reaction rates at room temperature were measured spectrophotometrically as indicated in Fig. 1. For each nucleotide the rates are expressed relative to the first value shown.

	Concn. of NH_3 (N)	Concn. of OsO_4 (mM)	Concn. of nucleotide (mM)	Relative reaction rate
Thymidylate	0·1	0·58	0·06	(1·0)
	0·1	0·29	0·06	0·48
	0·4	0·59	0·06	6·3
Deoxycytidylate	0·1	11·4	0·64	(1·0)
	0·4	5·7	0·64	2·1
	0·4	11·4	0·64	4·2
	0·4	22·8	0·32	4·2

Action of alkali on oxidized pyrimidine nucleosides and nucleotides. After oxidation of thymidine or thymidylate with osmium tetroxide, incubation with potassium hydroxide facilitates the reaction of the reactive C-1 group of the deoxyribose. Unfortunately, analysis of the products is complicated by the accompanying reduced forms of osmium tetroxide, which interfere in the colorimetric estimation of deoxyribose by the diphenylamine reaction (Burton, 1956). However, these materials are not present in sufficient quantities to interfere in the periodate–thiobarbituric acid method for deoxyribose (Waravdekar & Saslaw, 1959), and by suitable methods it is also possible to follow the release of P_i from oxidized nucleotides when they are degraded by diphenylamine and formic acid (Burton, 1956; Burton & Petersen, 1960). Some results obtained in these types of experiment are shown in Table 2. Thymidylate releases its P_i after successive treatments with osmium tetroxide, alkali and diphenylamine in aqueous formic acid. However, when thymidine is oxidized and treated with alkali, it does not yield deoxyribose quantitatively in the periodate–thiobarbituric acid reaction unless the solution is treated with dilute acid before the reaction with periodate (Table 2). This behaviour indicates that, after the treatment with alkali, an acid-labile group is still attached to C-1 of the deoxyribose, and suggests that the expected ureidoglycoside is indeed formed (cf. Cohn & Doherty, 1956). Further confirmation of this mechanism was given by the fact that the solution gave a colour reaction for urea (Coulombe & Favreau, 1963) and a fluorimetric reaction for acetol (Roberts & Friedkin, 1958).

The time-course of phosphate release in the diphenylamine and formic acid mixture (Fig. 5) is similar to that from deoxyadenylate or deoxyguanylate and slower than that from apurinic acid (Burton & Petersen, 1960).

On prolonged treatment at 0°, deoxycytidylate may be completely oxidized by osmium tetroxide, but subsequent treatment with 0·1 N-potassium hydroxide for 2 or 4 hr. at 37° followed by reaction with diphenylamine and formic acid for 15 hr. at 30° only released 25% of the phosphate as P_i. The amounts of P_i released from thymidylate and deoxycytidylate are compared in Fig. 6 after reaction with osmium tetroxide for various periods: the eventual release of P_i parallels the initial effect of the osmium tetroxide on the u.v. absorption of the nucleotides.

Degradation of pyrimidine oligonucleotides and of DNA. As a result of the findings of the above experiments with the monopyrimidine nucleotides, several oligonucleotides of the series $(C_nT)p_{n+2}$ were treated with osmium tetroxide and the products were examined by paper electrophoresis at pH 2·7, in some cases followed by chromatography at right angles to the direction of electrophoresis. These separations do not resolve the sequential isomers of

Table 2. *Action of alkali on thymidine and thymidine 5′-phosphate that had been oxidized by osmium tetroxide*

The compounds were treated with 7 mM-OsO_4 in 0·1 N-NH_3 for 50 min. Excess of reagent was removed by ether extraction, and 2 N-KOH was added to give a final concentration of 0·1 N. Duplicate samples were removed from the thymidine reaction mixture into 0·3 vol. of 2 N-HCl. One of the samples (*A*) was diluted with 5 ml. of water and stored at 4°; the other (*B*) was heated at 70° for 15 min. before dilution with water. Deoxyribose was then determined in both samples by the periodate–thiobarbituric acid reaction (Waravdekar & Saslaw, 1959). Samples from the thymidine 5′-phosphate reaction mixture were added to 2 vol. of 3% (w/v) diphenylamine in 98% (v/v) formic acid. Inorganic and organic phosphate were determined after incubation at 30° for 17 hr.

Duration of KOH treatment (min.)	Deoxyribose released from thymidine (% of theoretical)		P_i released from thymidine 5′-phosphate (% of total phosphate)
	No acid treatment (*A*)	After acid hydrolysis (*B*)	
0	5	20	15
30	25	72	68
60	26	80	91
90	30	94	93
120	31	96	93

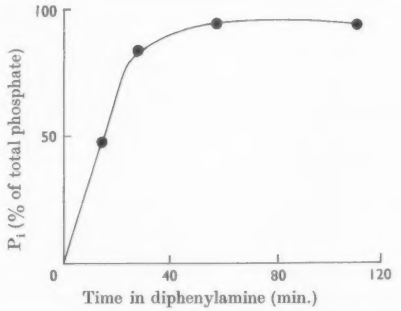

Fig. 5. Time-course of the diphenylamine-induced release of phosphate from thymidylate after treatment with OsO_4 and alkali. Thymidylate was treated with 7 mM-OsO_4 in 0·1 N-NH_3 at 0° for 45 min. and then with 0·1 N-KOH at 37° for 90 min. The solution was mixed with 2 vol. of 3% (w/v) diphenylamine in 98% (v/v) formic acid at 30°. Organic phosphate and total phosphate were measured at different times.

Table 3. *Release of cytosine nucleotides from oligonucleotides of formula* $(C_nT)p_{n+3}$

The oligonucleotides (2 mM) were treated with 7 mM-OsO_4 in 0·4 N-NH_3 at 0° for the times shown, excess of OsO_4 was removed by extraction and the solution was incubated at 37° for 2 hr. with 0·05 vol. of 2·1 N-KOH. The solution was desalted by treatment with Amberlite CG-50 (NH_4^+ form) and freeze-drying and then incubated with 2% (w/v) diphenylamine in 66% (v/v) formic acid at 37° for 1 hr., except in the one instance indicated. The products were separated and compared with authentic C_np_{n+1} compounds by paper electrophoresis at pH 2·7 (Petersen, 1963) followed in some cases by chromatography at right angles in propan-2-ol–water (3:2 or 7:3, v/v). Residual oligonucleotide includes material that appears to have been modified without breaking the internucleotide bonds.

		Residual oligonucleotide		Distribution of cytosine among products detected (%)			
Oligonucleotide	Time with OsO_4 (min.)	Amount (% of u.v. absorption at 267 mμ eluted)	E_{280}/E_{260} ratio	Cp_2	C_2p_3	C_3p_4	C_4p_5
$(CT)p_3$	5	29	1·4	100	—	—	—
	11	11·5	1·9	100	—	—	—
$(C_2T)p_4$	7	11	1·9	49·5	50·5	—	—
	10	6–9		50	50	—	—
	11	20	2·0	49·5	50·5	—	—
	15	10		54	46	—	—
	40	6		57	43	—	—
$(C_3T)p_5$	6	18	2·0	40	34	26	—
	6*	8·5	2·0	39	34·5	26·5	—
$(C_4T)p_6$	7	15	1·95	33·5	24·5	22	20

* Treated with diphenylamine and formic acid for 17 hr. at 30°.

isomeric $(C_nT)p_{n+2}$ mixtures. During treatment with osmium tetroxide in 0·4 N-ammonia, the E_{280}/E_{260} ratio (measured at pH 1–2) increased as the thymine residue reacted. In each case, only one ultraviolet-absorbing spot (E_{280}/E_{260} ratio about 2 at pH 1–2) was detected and it had virtually the same electrophoretic mobility as the original isomeric nucleotides, indicating that the internucleotide linkages were not affected. The treatment with potassium hydroxide slightly decreased the mobility but again only one component was seen. After treatment with diphenylamine, the expected C_np_{n+1} products were found (Table 3), and, in addition, there was some material with mobilities similar to those of the original oligonucleotides but with a higher E_{280}/E_{260} ratio. Apparently the thymine had been oxidized but the internucleotide bonds had not been broken. The incomplete cleavage was principally due to the short time of reaction with diphenylamine [see the second experiment with $(C_3T)p_5$ in Table 3] and so, in all subsequent experiments, the diphenylamine reaction was carried out at 30° for at least 15 hr.

Table 3 also shows that the reaction with osmium tetroxide was not complete at 5 min. but was essentially complete after about 7 min. in 0·4 N-ammonia at 0°. As expected, pCp was the principal u.v.-absorbing material present after degradation of the mixed isomers pCpTp and pTpCp. The expected products were also given by each of the higher homologues, e.g. $(C_2T)p_4$ gave pCp and pCpCp. It is noteworthy that $(C_4T)p_6$ gave a good yield of C_4p_5. Though these results are those to be expected from a completely specific removal of the thymidine residues from the original oligonucleotides, closer examination shows that there has also

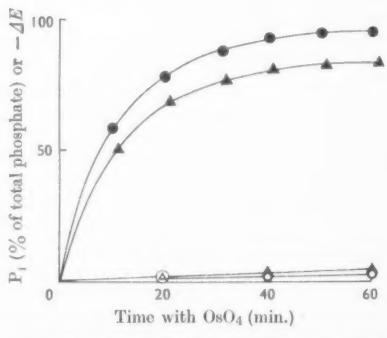

Fig. 6. Effects of different times of incubation with OsO_4. Thymidylate or deoxycytidylate (2 mM) was treated with 7 mM-OsO_4 in 0·1 N-NH_3 at 0°. After the removal of excess of OsO_4, samples were diluted in 0·1 N-HCl and the percentage fall of extinction coefficient ($-\Delta E$) was determined at 267 mμ for thymidylate (▲) and at 280 mμ for deoxycytidylate (△). Other samples were incubated for 2 hr. with 0·1 N-KOH at 37° and then in 2% (w/v) diphenylamine in 66% (v/v) formic acid for 12 hr. at 30°. P_i (as % of total phosphate): ●, thymidylate; ○, deoxycytidylate.

been some degradation of cytosine nucleotides, as is to be expected from the kinetics of the reaction of osmium tetroxide with nucleosides and mononucleotides. For example, more pCp than pCpCp was released from $(C_3T)p_5$, whereas these products should appear in equimolar amounts because they should come only from the isomers pCpCpTpCp and pCpTpCpCp. The actual ratio was about 1·2:2. Similarly, $(C_4T)p_6$ gave 1·5 times as much pCp as C_3p_4 when equimolar amounts were again expected. After degradation of $(C_2T)p_4$, the ratio of pCp to pCpCp increased with the time of reaction with osmium tetroxide. Likewise, the amount of P_i increases with the reaction time and eventually goes above the theoretical limit of 33% with $(CT)p_3$ (Fig. 7). These effects could be explained by oxidation of about 1% of the deoxycytidine residues/min. (in 0·4 N-ammonia). Fig. 7 also shows that heated DNA reacts readily with osmium tetroxide, whereas native DNA is slowly and incompletely attacked.

In other experiments (Tables 4 and 5) diphenylamine digests of herring DNA were treated with osmium tetroxide, and the cytosine nucleotides that were eventually liberated were separated on paper by two-dimensional procedures. For the experiments in Table 4 the digest was treated with prostatic phosphomonoesterase as described by Burton & Petersen (1960) before reaction with osmium tetroxide. In this case, the amounts of CpC and CpCpC nucleotides should not have been affected by the osmium treatment and, within the analytical errors of the experiment, this appears to be so. Appreciable amounts of several oligonucleotides containing one thymine nucleotide residue were also observed among the products in these experiments and indicate that the conditions of oxidation were not satisfactory.

Recommended conditions for selective degradation. The experiments in Fig. 7 and Tables 4 and 5 show that satisfactory results may be obtained by the following procedure. The oligonucleotide to be degraded is dissolved in 0·4 N-ammonia so that 1 ml. of the solution contains about 2 μmoles of thymine. The solution is cooled to 0° and osmium tetroxide (0·05–0·2 M) is added to give a final concentration of 7 mM. We consider that it is important to check each sample of osmium tetroxide by determining the rate of reaction with thymidylate. The E_{287} value after ether extraction should fall to 50% of the initial value in 10–11 min. in 0·1 N-ammonia. One sample that reacted at about half this rate

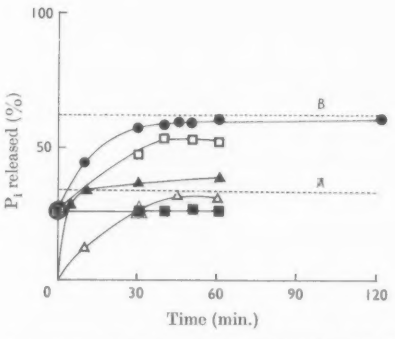

Fig. 7. Release of P_i from oligonucleotides and DNA treated with OsO_4. Concentrations were adjusted so that 1 ml. of nucleotide solution contained 2 μmoles of thymine. Solutions were then treated at 0° with 7 mM-OsO_4 in 0·1 N- or 0·4 N-NH_3 as indicated. At different times, samples were shaken with ether to remove excess of OsO_4, incubated with 0·05 vol. of 2·1 N-KOH for 2 hr. at 37° and then mixed with 2 vol. of 3% (w/v) diphenylamine in 98% (v/v) formic acid and incubated at 30° for 15–17 hr. ●, Diphenylamine digest of herring DNA; □, heat-denatured herring DNA; ■, native herring DNA; △, $(CT)p_3$; ▲, $(CT)p_3$ treated with OsO_4 in 0·4 N-NH_3. Except for the last series of values 0·1 N-NH_3 was used. The broken horizontal lines *A* and *B* show theoretical maximum yields from $(CT)p_3$–diphenylamine and DNA–diphenylamine digests respectively. These yields were calculated assuming no degradation of cytosine and, for *B*, assuming a random arrangement of nucleotides in the DNA chains.

Table 4. *Products obtained by treating a diphenylamine digest of DNA with prostatic phosphatase followed by successive treatments with osmium tetroxide, alkali and acidic diphenylamine*

A diphenylamine digest of herring DNA was treated with prostatic phosphatase. After removal of protein the product was treated with OsO_4 for 45 min. in 0·1 N-NH_3 at 0°. The treatments with alkali and acidic diphenylamine are described in the legend to Fig. 7. The mixture was then subjected to two-dimensional separation on paper, according to either Burton & Petersen (1960) or Petersen (1963). Individual values are shown. As the amounts of C_nP_{n-1} compounds should show no change, some data from Burton (1960) are included for comparison.

Product	Amount (moles of cytosine/100 moles of DNA phosphate)	Amount expected for randomly arranged polynucleotide	Burton (1960)
C	5·3, 5·7, 6·2	5·35	5·9
Cp	3·7, 4·8*	6·09	
Cp_2	1·0, 1·1, 1·1	1·76	
C_2p	1·4, 1·5, 1·8	2·20	1·8
C_2p_2	1·1, 1·5, 1·6	2·55	
C_3p_2	0·5, 0·5	0·69	0·64

* Corrected for 0·7 mole of deoxycytidine found as undegraded (CT)p.

Table 5. *Products obtained by treating a diphenylamine digest of DNA with osmium tetroxide followed by successive treatments with alkali, acidic diphenylamine and prostatic phosphatase*

A diphenylamine digest of herring DNA was treated with OsO_4 for 45min. in 0·1 N-NH_3. The treatments with alkali and acidic diphenylamine are described in the legend to Fig. 7. The mixture was then treated with prostatic phosphomonoesterase before two-dimensional separation of the nucleotides on paper, according to either Burton & Petersen (1960) or Petersen (1963). Individual values are shown.

Product	Amount (moles of cytosine/100 moles of DNA phosphate)	Amount expected for randomly arranged polynucleotide
C*	10·3, 10·9, 11·1, 11·4	13·1
C_2p	3·3, 3·5, 3·6, 3·7	5·5
C_3p_2	1·0, 1·1, 1·2, 1·5	1·73
C_4p_3	0·4, 0·4, 0·5	0·48

* Includes 0·3–0·7 mole for deoxycytidine found as (CT)p resulting from incomplete oxidation by the OsO_4.

also gave incomplete release of P_i, even when the initial oxidation stage had been taken to completion.

With a satisfactory sample of osmium tetroxide, the reaction is allowed to proceed for 6–7min. in 0·4 N-ammonia. The reaction is then stopped by extracting excess of osmium tetroxide three times with 5 vol. of ice-cold ether. Potassium hydroxide (2 N) is added to the aqueous phase to give a final concentration of 0·10 N. After 2hr. at 37°, the solution is desalted (see the Methods section) and the freeze-dried material is incubated for 15–20hr. at 30° in a suitable volume of 2% (w/v) diphenylamine in 66% (v/v) formic acid. An equal volume of water is added, the diphenylamine and most of the formic acid is removed by ether extraction and, if necessary, the residual formic acid is removed by exhaustive extraction with ether or by evaporation *in vacuo*.

DISCUSSION

In this work, our aim has been to degrade selectively pyrimidine-containing oligodeoxynucleotides by oxidation at the ethylenic bond of the pyrimidine. Osmium tetroxide reacts much faster with thymine nucleotides than with cytosine nucleotides and its selectivity is retained on reaction with oligonucleotides. It is most likely that the pyrimidines condense with the tetroxide to form the 4,5-diolosmates and that the subsequent treatment with alkali opens the pyrimidine rings so that they form acid-labile ureidoglycosides. In this way it is possible to remove nucleotides almost quantitatively. Provided that correction is made for the unwanted degradation of cytosine compounds (5–10%), the conditions described provide a potentially valuable method for investigating nucleotide sequences in DNA. The method may be particularly useful in examining sequences of pyrimidine deoxynucleotides such as those that have been isolated by Hall & Sinsheimer (1963) from the DNA of bacteriophage ϕX174.

If the oligonucleotides have been treated with phosphomonoesterase before reaction with osmium tetroxide, any terminal cytosine sequences appear as nucleotides of the general formula C_np_m, whereas interior cytosine nucleotides appear as C_np_{n+1} compounds. By suitable enzymic methods it should be possible to decide which of the C_np_n products have 3′-phosphate and which have 5′-phosphate end groups. With this information one could determine the sequence of any oligodeoxynucleotide that consisted of any number of cytosine nucleotide residues and up to two thymine nucleotide residues. The same procedure applied to a diphenylamine digest of DNA would give values for the frequencies of the various sequences Pu-C_n-Pu, T-C_n-Pu and Pu-C_n-T.

A disadvantage of the method is that the coloured colloidal reduced forms of osmium tetroxide are not easily separated from the oligonucleotide products of the degradation. Chromatography or electrophoresis on paper have been more useful for eliminating these colloidal materials than has column chromatography on DEAE-Sephadex or Dowex 1.

Although purine nucleotides are not attacked by osmium tetroxide, the conditions discovered for removing the oxidized thymine residues are sufficiently acid to remove the purines as well. The reactions cannot therefore be used to remove the thymine while leaving purine nucleotides intact. The reaction between oxidized pyrimidine oligonucleotides and the diphenylamine–formic acid reagent is somewhat slower than the reaction of the mononucleotides. This is not surprising in view of the fact that in the same reagent DNA reacts more slowly than the purine mononucleotides.

The series of reactions described cannot be used for base-sequence studies on RNA because uracil nucleotides are oxidized at a rate intermediate between those of thymine and cytosine nucleotides, and because the treatment with alkali would cause extensive degradation of RNA. In other experiments we have tried to replace the incubation with alkali by treatment with periodate, but the reaction is slow and incomplete.

Various structural modifications appeared to alter the rate of attack by osmium tetroxide in an independent manner. From Fig. 1 and other data, the relative rates of oxidation by osmium tetroxide in 0·4 N-ammonia are, approximately: deoxycytidine, 1·0; cytidine, 1·8; deoxyuridine, 2·8; uridine, 4·5; 5-methyldeoxycytidine and 5-bromodeoxyuridine, 13; thymidine, 45. Thus the increase of

reaction rate is about 15 times for the introduction of a 5-methyl group, 5 times on introducing a 5-bromo substituent, 2·5–2·8 times on replacing a 6-amino group by a 6-oxo group and 1·6–1·8 times on introducing a hydroxyl group at the 2′-position of a deoxynucleoside.

A number of other selective reactions of the nucleotide bases in DNA have been described. Grossman & Greenlees (1961) have shown that dihydropyrimidine nucleotides differ in their lability to acid. Deoxycytidine and its nucleotides are hydrolysed selectively by heating at pH 6–7 (Adamiec & Shugar, 1959; Greer & Zamenhof, 1962). Hydroxylamine (Verwoerd, Zillig & Kohlhage, 1963) and hydrazine (Verwoerd & Zillig, 1963) can react selectively with different pyrimidine nucleotides. Potassium permanganate oxidizes cytosine, thymine and guanine nucleotides, but adenine is resistant. In this way, the distribution of adenine may be examined in DNA or in polydeoxynucleotides (Jones, Ross, Takemura, Thompson & Walker, 1964; Jones & Walker, 1964). Simon & Van Vunakis (1962, 1964) have shown that guanine is selectively photo-oxidized in the presence of methylene blue, but methods for the selective and quantitative removal of the oxidized guanine have not been described. In at least one instance (Jones *et al.* 1964) the reactions may occur readily with model compounds but only very slowly in polynucleotides.

We thank Mr N. F. Varney for his willing and skilled technical assistance. W. T. R. held a Medical Research Council Scholarship for Training in Research Methods. The work was aided by grants from the U.S. Public Health Service and the Rockefeller Foundation.

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The Incorporation of Thymidine into Deoxyribonucleic Acid in Mouse Fibroblasts Infected with Polyoma Virus

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(*Received* 31 *May* 1965)

1. Mouse-fibroblast cultures in the stationary phase of growth show an increased rate of [^{3}H]thymidine incorporation into DNA from 12 to 44hr. after infection with polyoma virus. 2. Intracellular virus progeny is first detected at about 24hr. after infection. 3. Calculations based on the [^{3}H]thymidine-incorporation data and direct measurements of the DNA content of the cell cultures indicate that the amount of the excess of DNA synthesized by the infected cell cultures corresponds to about 10% of their total DNA. 4. The mitotic index of the cell cultures at 40hr. after infection was significantly higher than that of non-infected control cells. 5. Possible interpretations of the stimulation of DNA synthesis observed in polyoma-infected cell cultures are discussed.

Studies on a number of animal cells infected with various DNA viruses have shown that, soon after infection, marked changes occur in the normal pattern of DNA synthesis of the cell (Ben-Porat & Kaplan, 1963; Green & Daesch, 1961; Ginsberg & Dixon, 1959; Newton & Stoker, 1958).

The present work was undertaken to study the effect of polyoma infection on the process of DNA synthesis in mouse-fibroblast cultures. It was found that in the stationary phase of growth these cell cultures show an increased rate of incorporation of [^{3}H]thymidine into DNA 12hr. after virus adsorption. Direct measurements of the total DNA content of the cultures showed that the enhanced rate of thymidine incorporation corresponds to an accumulation of DNA in the infected cultures that greatly exceeds the amount required for virus production.

Increased DNA synthesis in polyoma-infected cells has been observed previously by Minowada & Moore (1963) by radioautography, and by Dulbecco, Hartwell & Vogt (1965), who showed, in addition, that most of the newly synthesized DNA was host DNA.

In the present paper we report the results of experiments that confirm the previous observations and give additional data on some aspects of the problem of the determination of the amount of DNA synthesized by the infected cell cultures, the stimulatory effect on DNA synthesis of the addition of fresh medium to the cell cultures and the mitotic index of the cell cultures at different times after infection.

MATERIALS AND METHODS

Secondary cultures of mouse-embryo cells were prepared by seeding 2×10^6 cells in 100mm. Falcon plastic Petri dishes with 10ml. of reinforced Eagle medium (fourfold concentration of amino acid and vitamins) supplemented with 10% of calf serum (Smith, Freeman, Vogt & Dulbecco, 1960). Cell counts were performed in a Bürker chamber. The cells were incubated under a flow of humidified CO_2–air mixture for 48hr. After that time the medium was replaced with medium containing 10% of horse serum; 48hr. later the medium was removed and the cell cultures were infected by the addition of 0·25ml. of an S.E. polyoma suspension (originally obtained from Dr B. Eddy), containing 4×10^8 plaque-forming units/ml. (30 plaque-forming units/cell). The cells were incubated for 3hr., and then the inoculum was decanted and the same medium present before infection was again added to the cultures (see the Results section).

Virus titration was carried out, after the cells had been disrupted by an ultrasonic treatment, by plaque assay as described by Winocour & Sachs (1960).

For the thymidine-incorporation experiments, 5μc of [^{3}H]thymidine, of specific activity 4c/m-mole (The Radiochemical Centre, Amersham, Bucks.), was added to the cultures. Incubation was stopped after 1hr. by adding 0·3ml. of aq. $HClO_4$ (final concn. 0·2N). Subsequent operations were performed at 2–4°. The cell residues were scraped off with a rubber 'policeman', transferred to a centrifuge tube and spun for 5min. at 2000*g*. The sediment was washed twice with 0·2N-$HClO_4$ and then once in ethanol and once with ethanol–ether (3:1, v/v). Finally, the sediment was suspended in 2ml. of 0·6N-$HClO_4$ and heated at 70° for 20min. On a portion of this hydrolysate the DNA content of the cell cultures was determined by using the diphenylamine method of Burton (1956). The ^{3}H-radioactivity was determined on another portion of the same

solution by using a Tracerlab liquid-scintillation counter, model LSC–20, an appropriate internal standard being used to allow for correction of the quenching caused by the acid solution. The efficiency of scintillation counting was 5%.

The mitotic index of the cultures was determined by counting all mitotic figures on seven or eight different cell preparations. Groups of 1000 cells were examined for each cell preparation. Bouin's fixative and haematoxylin–eosin staining were used.

RESULTS

Growth pattern of mouse-fibroblast cultures. The number of cells adherent to the plastic surface at 24hr. after seeding was about 1×10^6 and increased up to 4×10^6 by 72hr., then slightly decreased.

The DNA content of the cultures increased lineally from 24 to 72hr., whereas the specific ^{3}H-radioactivity of DNA decreased and then remained practically constant at very low levels (Fig. 1). The rapid fall of the curve of the ability to incorporate thymidine at the time when DNA synthesis is still going on is for the greater part the result of the increasing DNA content of the cultures. The low levels of the second part of the curve of the ability to incorporate thymidine is in accord with the extremely limited DNA synthesis occurring in the cultures after 72hr. The experiment reported in Fig. 1 shows that, from 24 to 48hr. after plating, the DNA content of the cultures increases by about 18μg. On the basis of the thymidine-incorporation data, it was calculated that, during the same period, only 0·27μg. of [^{3}H]thymidine can be incorporated into DNA. As this amount of thymidine corresponds to that contained in 1·3μg. of DNA, it appears that the coefficient of utilization of the exogenous [^{3}H]thymidine for DNA synthesis in our system is only 7·3%. This low thymidine utilization is not due to a lack of precursor, as during the experiment large amounts of [^{3}H]thymidine remain in the medium. Since it is known that, in mammalian cells, most of the DNA thymine is derived from the endogenous deoxyuridylic acid (Humphreys & Greenberg, 1958), this result is not surprising.

Effect of the addition of fresh medium to the cell cultures. During the present experiments it was noted that the simple replacement of the medium of the cell cultures with new medium, as is generally carried out after virus infection, produced a marked stimulation of the incorporation of [^{3}H]-thymidine into DNA for a period of a few hours.

This effect is shown in the experiment reported in Fig. 2, in which the rate of incorporation of [^{3}H]thymidine into DNA in cell cultures at different times after plating was measured. The medium was not changed in the cultures for 140hr., and then at 140 and 240hr. the medium was replaced with fresh medium.

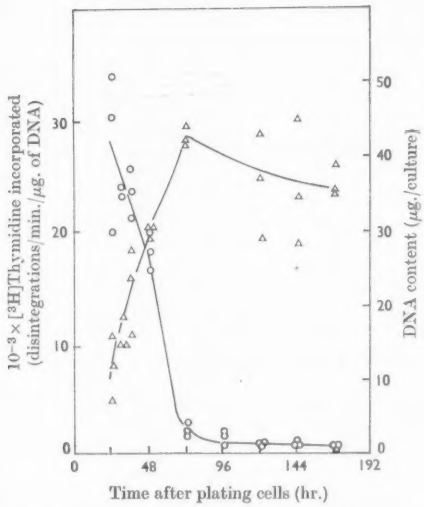

Fig. 1. Incorporation of [^{3}H]thymidine into DNA (○) and the total DNA content (△) in mouse-fibroblast cultures as a function of the cell age. Experimental details are given in the text.

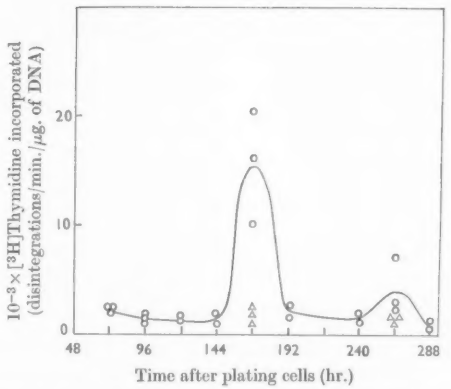

Fig. 2. Effect of replacing the medium in mouse-fibroblast cultures in the stationary phase of growth. ○, Specific ^{3}H-radioactivity of DNA of cell cultures labelled with [^{3}H]thymidine at the times noted; at 140 and 240hr. after plating the medium of the cell cultures was replaced with fresh medium. △, Specific ^{3}H-radioactivity of DNA of cell cultures in which at 140 and 240hr. the medium was replaced with medium obtained from 72hr.-old cultures. Experimental details are given in the text.

At 24hr. after the replacement of the medium, the rate of [^{3}H]thymidine incorporation into DNA was significantly increased. On the other hand, if the replacing medium was obtained from a 72hr. or older culture, no rise of the thymidine-incorporation rate occurred. The reason for the stimulating effect of fresh medium was not investigated further.

Experiments with polyoma-infected cells. On the basis of the results reported above, it was decided to replace the medium of the cultures 48hr. after plating and to infect the cells 48hr. later, when the rate of [^{3}H]thymidine incorporation is again low. After the virus was adsorbed, the same medium present in the cultures before infection was again added to the cell instead of fresh medium.

Control samples were handled in exactly the same way except that a non-infectious cell lysate was added instead of virus. The results of one experiment are shown in Fig. 3. In comparison with the control samples, the infected cell cultures show a marked increase in the rate of thymidine incorporation into DNA between 12 and 44hr. after infection.

Assuming that the coefficient of utilization of the exogenous thymidine in this cell system does not change as a consequence of the virus infection, it was calculated that, between 12 and 44hr. after the infection, about 3·3μg. of extra DNA was synthesized in each infected culture. These results were confirmed by direct determinations of the DNA content of the cell cultures in a number of samples sufficiently large to permit the statistical evaluation of the results (Table 1). At 24hr. after infection, the DNA content of infected cultures was about 4μg. greater than that of normal cultures. No further increase in the DNA content of the infected cell cultures was detected at 44hr. after infection. However, from 26 or 28hr. after infection some cells start to release virus particles and

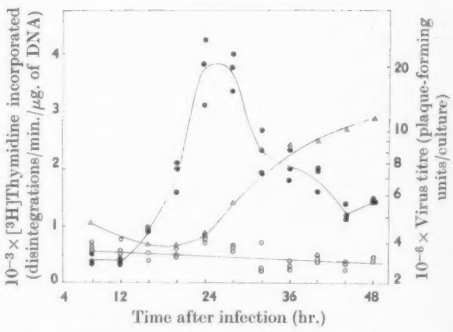

Fig. 3. Incorporation of [^{3}H]thymidine into DNA in mouse fibroblasts infected with polyoma. ○, Control cells; ●, infected cells; △, virus titre.

Table 1. *DNA contents of normal and polyoma-infected mouse-fibroblast cultures*

Experimental details are given in the text. The DNA contents are given as means ± S.D.

Time after infection (hr.)		No. of determinations	DNA content (μg./culture)	Significance of difference (Student's t test)
24	Normal cultures	20	42·79±4·06	$t=3·42$
	Infected cultures	20	46·20±3·09	$0·01>P>0·001$
44	Normal cultures	20	43·37±3·05	$t=4·38$
	Infected cultures	20	47·50±2·90	$0·01>P>0·001$

Table 2. *Mitotic indexes of normal and polyoma-infected mouse-fibroblast cultures*

Experimental details are given in the text; 1000 cells were counted for each determination. The mitotic indexes are given as means ± S.D.

Time after infection (hr.)		No. of determinations	Mitotic index	Significance of difference (Student's t test)
20	Control cultures	7	0·60±0·200	$t=2·26$
	Infected cultures	7	0·40±0·129	$0·05>P>0·02$
40	Control cultures	8	0·52±0·138	$t=5·133$
	Infected cultures	8	0·81±0·180	$0·01>P>0·001$

to lyse, and it is possible that after that time a part of the DNA present in these cells is degraded.

Intracellular virus started to form, as judged from the plaque assay, at about 24hr. after the infection.

Mitotic index of the cell cultures. The results of this study are shown in Table 2. It appears that at 20hr. after infection the mitotic index of the infected cultures was slightly lower than that of the control cultures. On the other hand, at 40hr. after infection the mitotic index of the infected cell cultures was significantly higher than that of the control cells.

DISCUSSION

The present results indicate that mouse-fibroblast cultures in the stationary phase of growth show a marked increase in the rate of DNA synthesis from 12 to 44hr. after infection with polyoma virus. Calculations based on the results of experiments on the incorporation of [^{3}H]thymidine into DNA suggest that about 3·3μg. excess of DNA is synthesized in the infected cultures.

As the virus may have altered the permeability of the cell membrane in some way, the results of experiments based on the incorporation of exogenous precursors may not necessarily be meaningful. It was therefore important to confirm by direct measurements that there was a real increase of the DNA content in the infected cultures. The results of these experiments indicated that the excess of DNA present in the infected cultures was about 4μg., corresponding to 10% of their total DNA. From the data published by Crawford, Crawford & Watson (1962) on the DNA content of the virus particles and the efficiency of the plaque assay, the total amount of virus found in our experiments 48hr. after infection should not contain more than 0·01–0·02μg. of DNA.

Minowada & Moore (1963) by radioautographic experiments, and Dulbecco *et al.* (1965) by experiments on the incorporation of [^{3}H]thymidine and [^{32}P]phosphate into DNA, previously reported the occurrence of a stimulation of DNA synthesis in mouse-embryo cells and mouse-kidney cells infected with polyoma virus. Minowada & Moore (1963) concluded that this effect was related primarily to virus production. Dulbecco *et al.* (1965), on the other hand, showed that the labelled precursors were incorporated predominantly into cellular DNA and only for a minor part into viral DNA. These authors suggested that the virus may stimulate the synthesis of cellular DNA in the infected cell with a mechanism probably similar to that involved in the process of cell transformation.

This attractive hypothesis, however, does not convincingly account for the present results. It is known, in fact, that in our experimental conditions not more than 5% of the cells of the cultures are initially infected and produce the virus. The remaining cells are either infected abortively or are not infected at all. If the increase of DNA observed were due to the infected cells, we should expect to find in these cells a 20-fold increase of their DNA. Previous cytological experiments, however, failed to show any large increase of DNA in polyoma-infected cells (Leuchtenberger, Leuchtenberger, Stewart & Eddy, 1961).

It seems more probable that in our cell cultures the increased DNA synthesis observed was due to the non-infected cells or to the abortively infected cells, which were stimulated in some indirect way to synthesize DNA and to divide.

Further studies at cytological levels are required to decide which type of cell is really responsible for the increased DNA synthesis observed in polyoma-infected cell cultures.

This investigation was supported by U.S. Public Health Service Research Grant CA05544-04 from the National Institutes of Health, and by Grant SCOM5-2-05(28) from N.A.T.O., Scientific Affairs Division. We are very grateful to Dr Roger Weil for introducing us to some details of the technique of growing the polyoma virus.

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Studies on Myoglobin from the Finback Whale *(Balaenoptera physalus)*

PREPARATION, PHYSICOCHEMICAL AND IMMUNOCHEMICAL CHARACTERIZATION, DIFFERENTIATION FROM SPERM-WHALE MYOGLOBIN, AMINO ACID COMPOSITION AND END-TERMINAL ANALYSES

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(*Received* 30 *June* 1965)

1. Crystalline myoglobin was isolated from the skeletal muscle of the finback whale and fractionated, in its cyanmet form, into nine components (I–IX) by chromatography on CM-cellulose. Also in the cyanmet form, it was resolved into six components by electrophoresis on starch gel. Correspondence between the electrophoretic and chromatographic components was determined, and interconversion between components revealed by chromatography and electrophoresis. 2. The chromatographic myoglobin components were homogeneous in the ultracentrifuge. Molecular weights of certain components were determined by means of sedimentation equilibrium and by gel filtration on Sephadex G-100. Values from these two methods corresponded to the minimum molecular weight calculated from the iron content. 3. The spectral properties of the chromatographic components were investigated in the visible and the ultraviolet ranges. 4. The major components of finback-whale myoglobin and sperm-whale myoglobin showed almost identical spectral, electrophoretic and chromatographic behaviours, but had different infrared spectra. The infrared spectra of the corresponding apoproteins were almost identical. 5. Rabbit antisera to sperm-whale myoglobin component X cross-reacted with finback-whale myoglobin components V, VI and VII only about 30%. 6. The major chromatographic components of finback-whale myoglobin have identical amino acid compositions. The polypeptide chain contains 151 amino acid residues and its molecular weight is 17504. 7. The *N*-terminal end of the chain is:

Val-Tyr-(Thr or Gly)-Leu
1 2 3 4

Amino acids released from myoglobin by the action of carboxypeptidase A at different intervals were determined.

Myoglobin of the sperm whale is the best characterized member of the myoglobin group. Its three-dimensional structure in the crystalline form has been deduced from X-ray crystallographic studies (Kendrew *et al.* 1960, 1961), and a great deal is known about its physical and chemical properties (Atassi, 1964*a*; Edmundson & Hirs, 1962*a*), its primary structure (Watson & Kendrew, 1961; Šorm, 1962; Edmundson & Hirs, 1962*a*,*b*,*c*; Edmundson, 1963, 1965) and its immunochemical properties (Atassi, 1964*a*, 1965*a*). It was therefore decided to isolate and study another myoglobin from a closely related species of whale and to determine its chemical composition and the effect of any slight variation in composition and primary structure on the physical, chemical and immunochemical properties of the haemoprotein.

The present paper deals with the preparation of myoglobin from the finback whale. Its chromatographic and electrophoretic heterogeneities have been studied and are discussed. Its spectral properties in the visible, ultraviolet and infrared regions have been studied and its immunochemical reaction with the antibody to sperm-whale myoglobin X (Atassi, 1964*a*) has been investigated. The molecular weight, amino acid composition and end terminal groups of the chromatographic fractions have been determined. The amino acid composition

of finback-whale myoglobin is compared with the compositions of other myoglobins.

MATERIALS AND METHODS

All experiments were carried out at 0–2°, unless otherwise stated.

Preparation and chromatography of myoglobin. Myoglobin was isolated from the skeletal muscle of the finback whale by the procedure of Perkoff & Tyler (1958) and crystallized twice by the method of Luginbuhl (1960). Spectral analysis of these preparations in the range 700–330 mμ showed that the myoglobin was in the ferri- or met-form. This was also observed when similar methods were used for human myoglobin (Perkoff, Hill, Brown & Tyler, 1962) and for SPMb* (Atassi, 1964*a*).

To avoid complications that might arise from autoxidation of myoglobins (Snyder, 1963), and to eliminate any chromatographic heterogeneity that disappears in the presence of cyanide, as with haemoglobin (Clegg & Schroeder, 1959) and human myoglobin (Perkoff *et al.* 1962), the haemoprotein was chromatographed in its cyanmet form. For preparative work myoglobin solutions (approx. 2 g. in 25 ml.) were chromatographed on columns (3·5 cm. × 60 cm.) of CM-cellulose (Sigma Chemical Co., St Louis, Mo., U.S.A.), with gradient elution from pH 6·2 to 7·7 in 10 mM-phosphate buffer containing KCN (0·01%). One magnetically stirred mixing chamber (250 ml.) was used and the buffer in the reservoir changed manually after a peak, or a group of peaks, had emerged. Fractions (9–10 ml.) were collected at the rate of 18–20 ml./hr. The extinctions of the fractions were read at 280, 420 and 540 mμ in a Zeiss PMQ II spectrophotometer. For analytical work, CN-MetMb samples (10–20 mg. in 1–3 ml.) were chromatographed on small columns (0·9 cm. × 60 cm.) by using a 50 ml. mixing chamber and collecting 1·5–2 ml. fractions at the rate of 3–4 ml./hr. Fractions belonging to one component were combined, dialysed against 10 mM-phosphate buffer, pH 6·5, containing KCN (0·01%), and concentrated on short CM-cellulose columns (2·2 cm. × 10 cm.) by applying at pH 6·5 and then eluting with a similar buffer at pH 7·7. By this method 20–40-fold concentration of myoglobin solutions was obtained.

Analytical methods. Characterization of the chromatographic fractions was carried out by starch-gel electrophoresis, at room temperature in the discontinuous buffer system of Poulik (1957), in gels and buffer containing KCN (0·05%). Cyanide was used in the buffers, since Perkoff *et al.* (1962) showed that differences in electrophoretic mobilities between the fractions of human myoglobin disappeared when the latter were analysed in their CN-MetMb form. The gels were stained with Amido Black (Smithies, 1959) and with benzidine hydrochloride (Haut, Tudhope, Cartwright & Wintrobe, 1962). Spectral analyses in the range 230–700 mμ were carried out in a Cary model 14 spectrophotometer. Complement-fixation experiments were done according to the method of Mayer, Osler, Bier & Heidelberger (1948) in buffers containing KCN (0·01%). Single diffusion in 1% agar was by Oudin's (1948) technique, and double diffusion by the method of Ouchterlony (1949). The lines were considerably sharper when gels contained KCN (0·05%). Iron content was determined by the method of Dietrich & Schmitt (1937). Sedimentation studies were made at 20° on 0·3–0·8% solutions of the proteins in 10 mM-phosphate, pH 7·2, containing KCN (0·01%), in a Spinco model E analytical centrifuge, by using a red filter and infrared film. Molecular weights were determined by means of sedimentation equilibrium (Van Holde & Baldwin, 1958) in the same buffer at 25° (density, ρ, at 25°, 0·9986 by pycnometry) and a speed of 20410 rev./min. The time at equilibrium was 16 hr. The curves obtained were scanned on a shadowgraph. The partial specific volume, $\bar{v}$, of CN-MetMb, estimated from the amino acid composition of FMb-VII, was 0·724 ml./g. Molecular-weight determination on columns (2·5 cm. × 55 cm.) of Sephadex G-100 was by a method similar to that described by Andrews (1964). In the present work, however, the above 10 mM-phosphate buffer, pH 7·2, containing KCN (0·01%), was used instead of the tris hydrochloride buffer used by Andrews (1964). The following proteins were used to calibrate the column: horse-heart cytochrome *c*, bovine ribonuclease, SPMb, human serum albumin, ovalbumin, chymotrypsinogen, γ-globulin and catalase.

For amino acid analysis myoglobin samples were exhaustively dialysed against water, freeze-dried and dried to a constant weight over P_2O_5 *in vacuo* at room temperature. Portions (2–3 mg.) were then dissolved in 2·0 ml. each of constant-boiling HCl (triple-distilled), flushed with N_2, evacuated (the procedure repeated twice), sealed and heated at 110° for 22 or 72 hr. The hydrolysates were freed from excess of HCl on a rotary evaporator and assayed with an amino acid analyser similar to that described by Spackman, Stein & Moore (1958). The tryptophan content was determined spectrally by the method of Goodwin & Morton (1946).

Infrared spectroscopy. For infrared analyses, the proteins were dialysed extensively against water, freeze-dried and then dried to a constant weight in a vacuum desiccator over P_2O_5. The KBr was dried and kept at 110° over P_2O_5 in an evacuated Abderhalden drier. The protein (1 mg.) was mixed with KBr (200 mg.) for 2 min. in a Clevdent amalgamator. Several proportions of protein to KBr were tried and best resolutions were obtained with the ratio 1:200. Pellets were made with an evacuable die by using 200 mg. of mixture and 20000 lb./in.^{2} pressure for 1 min. (after evacuation for 15 min.). The pellets were scanned in a Perkin–Elmer 521 infrared spectrophotometer. A blank pellet of 200 mg. of KBr was used for the reference beam.

Preparation of globin. The apoproteins were prepared by a method similar to that described by Theorell & Akeson (1955).

Immunization. The preparation of rabbit antibodies to SPMb-X has been described by Atassi (1965*a*).

N-Terminal analyses by dinitrophenylation. The protein (30–40 mg.) was dissolved (or suspended) in 3% (w/v) $NaHCO_3$ (5 ml.), and then 2 ml. of 5% (v/v) fluorodinitrobenzene in ethanol was added. The mixture was stirred magnetically at room temperature for 2 hr. The insoluble DNP-protein was washed on the centrifuge three times each successively with water and ethanol. Excess of fluorodinitrobenzene was then extracted ten times with peroxide-free water-saturated ether and the mixture dried

* Abbreviations: SPMb, sperm-whale myoglobin; FMb, finback-whale myoglobin (when these are followed by Roman numbers, the latter indicate the number of the chromatographic component); CN-MetMb, cyanmetmyoglobin.

over P_2O_5 in a vacuum desiccator. Portions of the DNP-proteins were hydrolysed at 110° for 8, 12 and 22 hr. with 2 ml. of constant-boiling HCl in a sealed N_2-flushed evacuated container as described above. The hydrolysates were evaporated to dryness at 30° on a rotary evaporater, excess of dinitrophenol was removed by sublimation and the ether-soluble DNP-amino acids were removed by ten extractions with water-saturated ether from N-HCl. The combined ether extracts were backwashed three times with N-HCl and finally evaporated to dryness. The residue was examined quantitatively by paper chromatography with, in the ascending dimension, the toluene–chloroethanol–pyridine–aq. NH_3 solvent of Biserte & Osteux (1951), and 1·5 M-phosphate buffer, pH 6·0, in the second descending dimension. A duplicate preparation of the ether residue was also hydrolysed with saturated $Ba(OH)_2$ (2 ml.) for 90 min. at 100°. After removal of Ba^{2+} as $BaCO_3$ the amino acids in the hydrolysates were assayed on the automatic amino acid analyser.

The aqueous phase was evaporated to dryness and hydrolysed at 110° *in vacuo* with constant-boiling HCl, to bring the total time of hydrolysis to 22 hr., and subjected to quantitative amino acid analysis as described above.

Sequential N-terminal analyses by the Edman procedure. The following modification of the Edman (1953) procedure was employed. The procedure was carried out only on FMb-VII. Myoglobin solution in water (5 ml. containing 106 mg. of Mb) was mixed with an equal volume of peroxide-free dioxan, adjusted to pH 8·7–9·0 with 0·1 N-NaOH and stirred for 2–4 hr. with 0·5 ml. of phenyl isothiocyanate. The reaction was performed in a standard-joint 50 ml. centrifuge tube. During reaction, the magnetically stirred mixture was kept at 40° and pH maintained at 8·7–9·0 by the addition of 0·1 N-NaOH. When the reaction was complete the mixture was extracted seven times with 5 ml. of thiophen-free benzene; the tubes were centrifuged to break the emulsion that usually formed. The reaction mixture was evaporated to dryness on the rotary evaporator (28°) and kept in the desiccator under vacuum for 24 hr. over NaOH pellets and paraffin shavings. For cyclization the residue was suspended in 7 ml. of acetic acid–conc. HCl (5:1, v/v) (Acher, Laurilla & Fromageot, 1956), and kept at 40° for 2–6 hr. The cyclized mixture was evaporated to dryness, suspended in 7 ml. of 10 mN-HCl, and extracted twice with an equal volume of water-saturated ethyl acetate and five times with water-saturated ether. The combined ether and ethyl acetate extracts were backwashed three times with 5 ml. of 10 mN-HCl and finally evaporated to dryness. The residue, containing the phenylthiohydantoin amino acids, was hydrolysed *in vacuo* in 5·7 N-HCl (2 ml.) at 140° for 20 hr., and the amino acids in the hydrolysates were determined quantitatively on the automatic amino acid analyser as described above.

The aqueous layer in the reaction tube was evaporated to dryness. At this stage a portion (4 mg.) was taken for two duplicate amino acid analyses. The remainder was suspended in water (5 ml.) and dioxan (5 ml.) and the next step of the degradation procedure carried out.

Identification of C-terminal residues. This was carried out only on FMb-VII. Myoglobin (30·0 mg.), dissolved in water (24 ml.) that had been adjusted to pH 8·1, was incubated at 25° with 0·6 mg. of carboxypeptidase A (DFP-carboxypeptidase A, 3 × crystallized; Worthington Biochemical Corp., Freehold, N.J., U.S.A.). The pH was maintained at 8·0 by the addition of 10 mN-NaOH. Portions (4 ml. each) were withdrawn after 6, 16, 31, 60 and 120 min., the pH was adjusted immediately to 3·0 and the solutions were dialysed separately against eight 10 ml. changes of mN-HCl. The diffusates from the digests were each combined and freeze-dried separately. The amino acids in the freeze-dried residues were determined on the amino acid analyser.

RESULTS

Chromatographic and electrophoretic behaviour. Electrophoresis on cellulose acetate of solutions of crystalline FMb, in the met form, in barbiturate buffer, pH 8·6, and room temperature showed that the preparations were completely homogeneous. Electrophoresis of CN-MetMb on starch gel revealed considerable heterogeneity. At least six bands were obtained with mobilities: *A*, 0·32 ± 0·03; *B*, 0·50 ± 0·03; *C*, 0·59 ± 0·02; *D*, 0·73 ± 0·02; *E* (observed only when large quantities were applied), 0·85

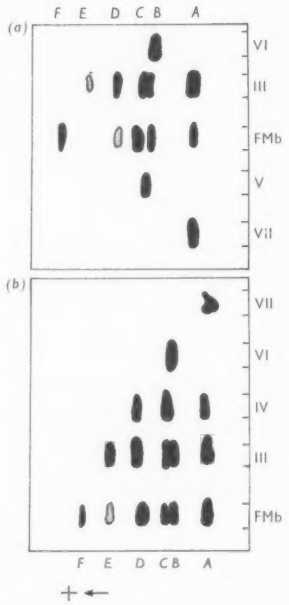

Fig. 1. Electrophoresis on starch gel of crystalline FMb and some chromatographic components. (*a*) Electrophoresis was carried out for 16 hr. at pH 8·6 (by the discontinuous buffer system of Poulik, 1957), with a potential gradient of 4·8 v/cm. The gels and buffer contained KCN (0·05%). (*b*) Electrophoresis was in the same buffer for 5 hr., with a potential gradient of 8·6 v/cm. The Roman numerals indicate the numbers of the chromatographic components. Electrophoresis bands are designated *A*–*F*.

$\pm 0{\cdot}01$; *F*, 1 (9–10cm. in 16hr.). Bands *A–E*, all visible as red spots before staining, were haemoproteins since they all gave a positive colour with benzidine, whereas band *F* failed to stain with benzidine. The electrophoretic pattern of unchromatographed crystalline FMb is shown in Fig. 1. Chromatography on CM-cellulose resolved the preparation into nine well-defined peaks (Fig. 2). The total recovery based on the extinction at 280mμ was 98·8%, and comprised: I, 14·2%; II, 2·31%; III, 12·3%; IV, 10·9%; V, 12·3%; VI, 6·67%; VII, 29·3%; VIII, 3·82%; IX, 6·30%.

The homogeneity of the chromatographic components was investigated by immediate electrophoresis on starch gel (Fig. 1). Components V, VI and VII were completely homogeneous and corresponded to bands *C*, *B* and *A* respectively. Component III was extremely heterogeneous and contained all bands *A*, *B*, *C*, *D* and *E*, with *D* being the major band. Component IV showed bands *A*, *C* (major band) and *D*. The non-haem protein I showed only slight traces of myoglobin bands *C* and *D*. The heterogeneity of components III and IV was confirmed by rechromatography on CM-cellulose. Thus component III was resolved into components that were eluted like components III, IV, V, VI, VII and VIII, whereas component IV was resolved into components III, IV, VI and VII. The fractions belonging to each of these peaks were combined, concentrated on CM-cellulose and again subjected to starch-gel electrophoresis and column chromatography. Components V, VI and VII were homogeneous by electrophoresis and by immediate chromatography. Components III and IV were again heterogeneous by both methods of analysis, component III being resolved into III, IV, V, VI and VII, and component IV into III, IV, V–VI and VII. This heterogeneity of components III and IV could not therefore arise from contamination with the other components. Rather, it was most probably due to the transformation of each of these two components into all the others. This transformation was by no means confined to components III and IV, since it could easily be demonstrated in components V, VI and VII when the latter were kept at 40° in 10mM-phosphate buffer, pH 7·0, containing cyanide (0·01%). The transformation was more effectively demonstrated by rechromatography of components V, VI and VII after dialysis against water and freeze-drying. Table 1 summarizes the results of these rechromatography experiments and suggests that the heterogeneities observed with components III and IV, and with the other components after certain physical

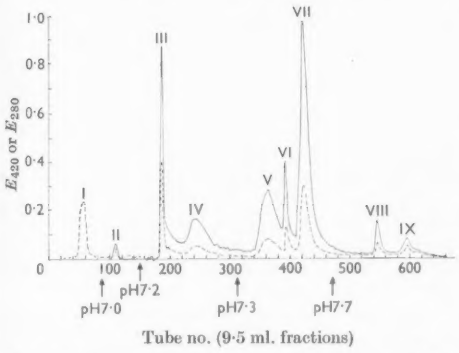

Fig. 2. Chromatogram of FMb on CM-cellulose. CN-MetMb was chromatographed on a column (3·5cm. × 60cm.) by gradient elution from pH 6·2 to 7·7 in 10mM-phosphate buffers containing KCN and employing one mixing chamber. The buffer in the reservoir was changed at the points indicated by arrows. ——, E_{420}; – – – –, E_{280}.

Table 1. *Rechromatography of FMb components*

The components were prepared by column chromatography. The effluent fractions belonging to one component were combined and concentrated on CM-cellulose. Experimental details are given in the text. They were then subjected to rechromatography immediately or after certain treatments. Each value represents the result of one chromatographic separation. Values were calculated [except for Expt. (*a*)] on the basis of E_{424}.

Expt.	Material and treatment — Component ...	Composition (%) I	II	III	IV	V	VI	VII	VIII	IX
(*a*)	FMb	14·2	2·31	12·3	10·9	12·3	6·67	29·3	3·82	6·30
(*b*)	III from (*a*)	—	1·8	49·9	23·9		12·1	12·0	1·2	<1
(*c*)	III from (*b*)	—	—	46·3	26·0		11·3	10·6	<1	<1
(*d*)	IV from (*a*)	—	—	6·8	78·7	<1	1·4	15·2	<1	<1
(*e*)	IV from (*d*)	—	—	5·2	83·6	—	1·0	9·1	—	—
(*f*)	VI from (*a*)	—	—	—	—	2·4	93·0	2·1	—	—
(*g*)	VI from (*a*) after freeze-drying	—	—	<1	1·2	10·3	76·1	8·3	—	—
(*h*)	VII from (*a*)	—	—	—	—	—	1·1	98·8	—	—
(*i*)	VII from (*a*) after freeze-drying	—	—	—	<1	12·2	4·1	82·1	—	—

treatments, must be due to transformation of each of these components into all (or most of) the others. The possibility that this transformation was caused by alkaline and acid metmyoglobin forms at equilibrium with each other, similar to that reported for human myoglobin (Perkoff *et al.* 1962), was excluded. This was because a large number of components had to be accounted for, and both chromatography and electrophoresis were carried out in the presence of excess of cyanide (under which conditions the heterogeneity of human myoglobin disappeared).

Fig. 3 shows the results of two chromatographic experiments of FMb-VII and that of the major SPMb component (i.e. component X; Atassi, 1964*a*) on the same CM-cellulose column (2·5cm. × 60cm.). Solutions were applied in 10mM-phosphate buffer, pH7·0, containing cyanide (0·01%), on a column that had been equilibrated with the same buffer. Gradient elution was employed, with one magnetically stirred mixing chamber containing 150ml. of the above buffer. When application of the myoglobin solution was complete, the reservoir was filled with a similar buffer but at pH7·32. Fig. 3 shows that SPMb-X and FMb-VII had very slightly different elution volumes, with FMb-VII being eluted first. The ratio of the volume required to elute FMb-VII over the volume required to elute SPMb-X was 0·910. Electrophoresis on starch gel showed that these two proteins had almost the same mobility, with FMb-VII occasionally moving very slightly ahead of SPMb-X.

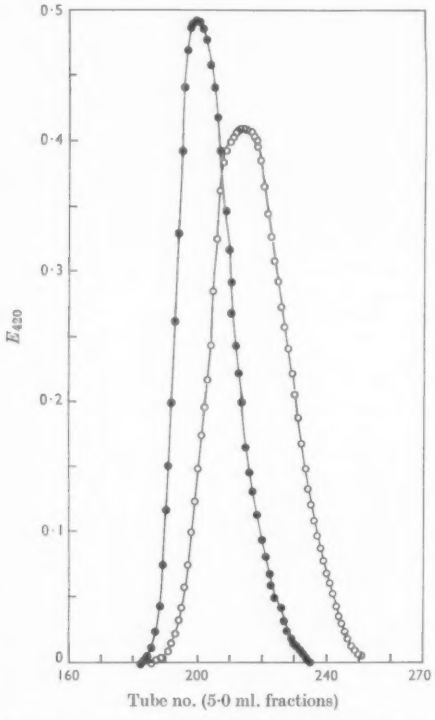

Fig. 3. Rechromatography of FMb-VII and SPMb-X. The CN-MetMb samples were chromatographed on the same CM-cellulose column (2·5cm.×60cm.). ●, FMb-VII; ○, SPMb-X. FMb-VII always tended to give sharper peaks than those given by SPMb-X. Experimental details are given in the text.

Spectral properties. Component I [in 10mM-phosphate buffer, pH7·2, containing cyanide (0·01%)] had an absorption maximum at 280mμ and a small maximum at 400mμ. The absorption at 400mμ was most probably due to contamination with myoglobin. This was confirmed by the results of starch-gel electrophoresis, since, as mentioned above, a small amount of material migrating like bands *C* and *D* was observed. Solutions of components II–IX, when analysed spectrally in 10mM-phosphate buffer, pH7·2, containing cyanide (0·01%), were in the CN-MetMb forms, showing maxima at 540, 424, 360–358 and 278–280mμ. Table 2 lists the ratios of the extinctions at the various absorption maxima to the extinction at 280mμ. The molar extinction coefficient of FMb-VII, on a dry-weight basis, was ϵ_{280} $32{\cdot}0\times10^3$. Component I had a very low E_{400}/E_{280} ratio (0·132), indicating that it was mostly (over 95%, assuming E_{424}/E_{280} 3·60 for 100% Mb) a non-haem protein. After rechromatography on CM-cellulose at pH6·2, the peak at 400mμ disappeared almost completely. Component II was also highly contaminated with

Table 2. *Ratio of extinctions at the absorption maxima of FMb components*

Proteins were prepared by one chromatography experiment, and their spectra determined at pH7·20 in 10mM-phosphate buffer, containing KCN (0·01%).

Component	E_{360}/E_{280}	E_{424}/E_{280}	E_{540}/E_{280}	E_{424}/E_{540}
I	—	0·132*	—	—
II	0·488	1·28	0·186	6·88
III	0·645	2·31	0·225	10·23
IV	1·19	3·91	0·381	10·19
V	1·04	3·82	0·362	10·57
VI	0·951	3·54	0·331	10·70
VII	0·949	3·61	0·339	10·64
VIII	0·828	2·78	0·310	8·94
IX	0·617	1·77	0·170	10·38

* This value represents the E_{400}/E_{280} ratio, since component I showed a minor peak at 400mμ only.

non-haem protein, as shown in Table 2 (E_{424}/E_{280} 1·28). Component III represented the first of the myoglobin components, but was probably contaminated with colourless protein. However, such a conclusion based on E_{424}/E_{280} could be misleading, since this ratio might actually vary for the different chromatographic components, which could all be pure myoglobins. However, rechromatography of component III showed, as pointed out above, the presence of small amounts of component II (Table 1). Components IV–VIII had constant, almost identical, extinction ratios and probably represent pure myoglobins. These myoglobin components had identical amino acid compositions (see Table 5), thus confirming their purity. The absorption spectrum of component VIII (Fig. 4) was unique in that it showed in the ultraviolet region, in addition to a maximum at 280mμ, absorption maxima at 261·5, 256, 249 and 244mμ, most probably due to contamination or complex-formation with a low-molecular-weight compound. The significance of the occurrence of these maxima, in FMb-VIII only, is not understood.

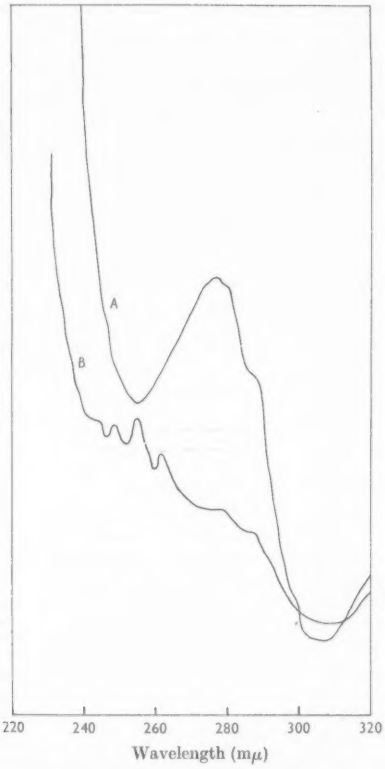

Fig. 4. Spectra of freshly prepared FMb-VII (*A*) and FMb-VIII (*B*) in the ultraviolet region. Solutions (130mg. of Mb/ml.) were in 10mM-phosphate buffer, pH 7·2, containing KCN (0·01%). In addition to a maximum at 280mμ (given by both FMb-VII and FMb-VIII), FMb-VIII shows maxima at 261·5, 256, 249 and 244mμ, most probably due to contamination or complex-formation with a low-molecular-weight compound. The spectra are displaced vertically for clarity.

Molecular-weight measurements. The minimum molecular weights calculated from the iron content of the twice-chromatographed components are shown in Table 3. The average value for the molecular weight, assuming 1 haem group/mol., was 18120. The possibility that the electrophoretic and chromatographic heterogeneities of FMb, and the intertransformation of the components, might arise from the presence of different aggregates of the monomer was investigated in the ultracentrifuge. Components II, VIII and IX were not analysed, but the other components (all chromatographed twice) sedimented as single sharp peaks. The peaks obtained with components III–VII sedimented at the same rate when the solutions had the same concentration. The sedimentation coefficient, $S^0_{20,w}$, of component VII was 1·96s.

Table 3 also shows the results of the molecular-weight determinations of certain components by means of sedimentation equilibrium. The values for the weight-average molecular weights, M_w, and the number-average molecular weights, M_n, obtained in each case corresponded to the molecular weight of the monomer. Therefore an equilibrium involving different aggregates of the monomer was ruled out as being the cause of the electrophoretic and chromatographic heterogeneities. Molecular-weight determinations of certain components by gel filtration on Sephadex G-100 (Table 3) confirmed the results obtained with sedimentation equilibrium.

Infrared spectroscopy. The above reported differences in the chromatographic and possibly electrophoretic behaviours of SPMb-X and FMb-VII could not be considered as evidence that these two myoglobins are different. The first conclusive demonstration, by physical methods, of differences between the two proteins came from the infrared spectra of the crystalline proteins. Table 4 lists the principal absorption bands of SPMb and FMb. No significant differences at frequencies higher than 1800cm.$^{-1}$ were observed. The only differences observed in the region 2000–4000cm.$^{-1}$ were actually in intensity rather than position of the above group frequencies. FMb repeatedly gave spectra with bands that were not as strong or as sharp as the corresponding bands of SPMb.

Fig. 5 shows the spectra of FMb and SPMb in the range 250–2000cm.$^{-1}$. The amide I band (C=O

Table 3. *Molecular-weight determinations of FMb components*

Molecular weights were determined, on the twice-chromatographed components, by the different procedures mentioned below and are listed together to facilitate comparison.

	Procedure of molecular-weight determination				
	Iron content		Sedimentation equilibrium		Sephadex G-100
Component	Iron (%)	M	M_w	M_n	M
III	0·298	(18742)	(18817)	(18628)	—
IV	0·307	18192	17634	18220	—
V	0·306	18250	18000	17920	17800
VI	0·308	18133	18140	18500	18000
VII	0·312	17900	18040	18170	17800
Average M*...		18120	17950	18200	17870

* Values in parentheses are not included in the averages.

Table 4. *Principal absorption bands of SPMb and FMb*

—, Band absent; (s), strong; (m), medium; (w), weak.

Band position (cm.$^{-1}$)	
SPMb	FMb
3300 (s)	3300 (s)
3070 (w)	3070 (w)
2960 (e), 2928 (s), 2870 (w)	2960 (m), 2928 (m), 2870 (w)
1650 (vs)*	1650 (vs)*
1534, 1517, 1500* (vs)	1534, 1517, 1502 (vs)*
1468 (vw)	1468 (vw)
1450 (s)	1450 (vw)
1390 (s)	1391 (s)
1310 (vw)	1310 (s)
1260, 1235 (s)	—
1150 (m), 1105 (s)	—, 1095 (vs)
1075 (m)	—
1025–1045 (s)	—
—	980 (m)
930 (m)	915 (w)
—	848 (m)
695 (w), 660 (w), 615 (m)	698, 663 almost absent
—	532 (vs)

* Band showed splitting.

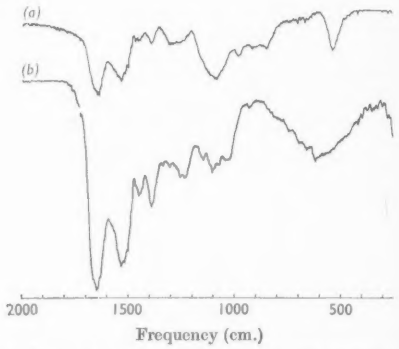

Fig. 5. Infrared spectra of FMb-VII (*a*) and SPMb-X (*b*). The spectra are given in the range 2000–250 cm.$^{-1}$. Above 2000 cm.$^{-1}$ the spectra of the two proteins were almost identical and are therefore not shown.

stretching) appeared in both proteins at 1650 cm.$^{-1}$. The group of bands at 1534, 1517 and 1500 cm.$^{-1}$ was due to the amide II band. The splitting observed in amide I and II vibrations was most probably due to vibrational coupling between neighbouring peptides (Miyazawa, 1960). The weak band at 1468 cm.$^{-1}$ was probably due to the CH_3 group frequency of alanine (Fraser, 1961), and the bands of 1450 and 1390 cm.$^{-1}$ were probably due to the C–CH_3 asymmetric and –$C(CH_3)_2$ symmetric deformation frequencies (Fraser, 1961). The five-membered ring stretching vibration at about 1400 cm.$^{-1}$ (strong in haemin) could also contribute to the band at 1390 cm.$^{-1}$. The pyrrolic C–N stretching frequency (Schwartz, Berg, Bossenmaier & Dinsmore, 1960) at 1340 cm.$^{-1}$, which was a very weak band in SPMb, disappeared completely in FMb. The first major change in the spectra was in the remarkable difference in the intensity of the band at 1310 cm.$^{-1}$ (CH_2 and CH_3 deformation frequencies; Fraser, 1961). This band was only a minor band in SPMb and appeared as a strong major one in FMb. The bands at 1260 and 1235 cm.$^{-1}$ in SPMb might be due to the amide III vibration. Also, at 1270–1230 cm.$^{-1}$ the C–O stretching frequency of acid groups on porphyrins appears (Schwartz *et al.* 1960). SPMb showed a distinct sharp band at 1150 cm.$^{-1}$ (CH_3 group frequency of alanine; Fraser, 1961; phenolic C–OH stretch; Jones, 1959) and another yet stronger band at 1105 cm.$^{-1}$ (bending and deformation of pyrrolic

NH; Schwartz *et al.* 1960). In FMb the 1150cm.$^{-1}$ band appeared only as a shoulder on the high-frequency side of the 1095cm.$^{-1}$ band. The next two bands at 1075 and 1035cm.$^{-1}$ (C–OH stretching; Zeiss & Tsutsui, 1953) were present in SPMb, but absent from FMb. A sharp band at 980cm.$^{-1}$ appeared in FMb and was absent from SPMb. This might be due to the =CH (or vinyl groups) bending and deformation that usually appears in porphyrins at 990cm.$^{-1}$ (Schwartz *et al.* 1960). FMb had a unique band of medium intensity at 848cm.$^{-1}$, probably due to CH out-of-plane vibrations as shown by pyrroles at 838cm.$^{-1}$ (Katritzky, 1959). Amides V and IV at 695 and 660 cm.$^{-1}$ respectively were much weaker in FMb. The amide VI vibration (C=O out-of-plane bending) at 615cm.$^{-1}$ disappeared completely in FMb, and a unique strong and sharp band appeared at 532cm.$^{-1}$. This new band might be due to a tremendous shift of amide VI. The spectra of the two apoproteins, although different from their parent myoglobins, were almost identical.

Immunochemical studies. On double diffusion in agar of FMb components V, VI and VII, together with SPMb-X (Atassi, 1964*a*), against rabbit antiserum to SPMb-X (Atassi, 1965*a*), the FMb components gave very faint lines as compared with that given by SPMb-X. In each case, a spur was formed, as a continuation of the SPMb-X line, indicating a partial similarity between SPMb and FMb. The extent of reaction of FMb components V, VI and VII with the antibody to SPMb-X was determined quantitatively by complement fixation. Although all myoglobins showed maximum complement fixation at the same antigen concentration (Fig. 6), the FMb components fixed at that maximum no more than one-third the amount of complement fixed by SPMb-X. The partial cross-reaction of FMb with anti-SPMb was reported qualitatively by Kendrew, Parrish, Marrack & Orlans (1954), who used gel diffusion methods.

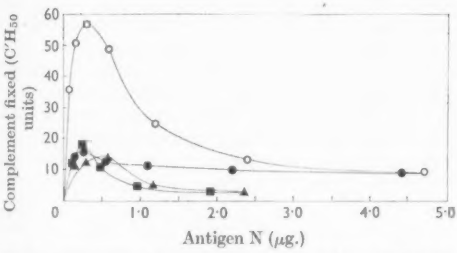

Fig. 6. Reactions of antibody to SPMbX with: ○, SPMb-X; ●, FMb-IV; ▲, FMb-V; ■, FMb-VII. Complement fixed ($C'H_{50}$ units) is plotted against the amount of antigen nitrogen.

Amino acid composition. Chromatographic components III, IV, VI, VII, VIII and IX of FMb, obtained after one chromatographic procedure, were subjected to amino acid analysis. Values were calculated on an ash-free and moisture-free basis. Amino acid contents are expressed as molar proportions of the stable amino acids: valine, glycine, aspartic acid, glutamic acid, leucine, alanine and lysine. The average molar proportions of these seven amino acids were (to the closest integer): 6:11:12:17:17:18:20. Treatment of the results is described in the footnotes of Table 5.

Table 5 shows the amino acid compositions of FMb components. The composition of FMb-III appeared to be slightly different from the composition of components IV–VII, the latter probably having identical amino acid compositions. The differences in the amino acid composition of FMb-III were probably due to contamination of this protein with some non-myoglobin protein. This was discussed above (one preliminary analysis indicated that the amino acid composition of component I was completely different from that of myoglobin). Slight differences in composition were also observed with components VIII and IX. This might have also been due to contamination. Although no differences in amino acid compositions could be observed with components IV–VII, there is some degree of uncertainty in some amide ammonia values with components that were subjected only to one hydrolysis time (e.g. FMb-IV). The molecular weight of components IV–VII, based on the amino acid composition and the nitrogen recovery, together with the contribution of the haem, was 17504.

Identification of the N*-terminal residues.* The *N*-terminus was determined for components I–VII by dinitrophenylation. Components III–VII, by quantitative paper chromatography of the ether-soluble fractions, gave DNP-valine as the major spot with recoveries 0·6–0·8mol. of valine/mol. of Mb. Amino acid analysis of the ether-soluble fraction of FMb-VII, after hydrolysis with barium hydroxide, revealed the presence of valine and leucine, with recoveries (in mol. of amino acid/mol. of Mb) 0·790 and 0·085 respectively. Amino acid analyses of the aqueous phases from the acid hydrolysates of the DNP-Mb components III–VII revealed in every case a decrease only in the valine value (as compared with the parent fraction) by 1mol./mol. of Mb. It was therefore concluded that the *N*-terminus for all components III–VII was valine. Qualitative paper chromatography revealed only glycine as the *N*-terminus of component I.

Edman degradation was carried out only on FMb-VII. Amino acids in the ether–ethyl acetate phase from the first cyclization step were (in mol. of amino acid/mol. of Mb): valine, 0·830; alanine, 0·238; glycine, 0·164; leucine, 0·092; aspartic acid,

Table 5. *Amino acid compositions of FMb components*

Values for FMb-VIII and FMb-IX were obtained from one analysis and are not therefore corrected. Values for FMb-III and FMb-IV are averages from two determinations of two 22hr. hydrolysates each. Values for FMb-VI are averages from two determinations each of 22, 24 and 72hr. hydrolysates (i.e. a total of six determinations). Values for FMb-VII are averages from five 22hr., one 24hr. and three 72hr. hydrolysates (i.e. a total of nine determinations). N.D., Not determined. Values in parentheses were not included in the averages.

	Amino acid composition (residues/mol.)									
			FMb-III		FMb-IV		FMb-VI		FMb-VII	
Amino acid	FMb-VIII	FMb-IX	Average	Nearest integer	Average	Nearest integer	Average	Nearest integer	Average	Nearest integer
Asp	12·4	12·9	13·5	14	12·1	12	12·15	12	12·20	12
Thr	6·10	4·74	5·25	5	5·01	5	5·38*	5	5·14*	5
Ser	6·29	6·06	4·78	5	4·92	5	5·14*	5	5·42*	5
Glu	16·6	17·1	16·2	16	16·6	17	16·57	17	17·05	17
Pro	4·38	5·37	5·34	5	4·09	4	3·77	4	4·00	4
Gly	11·1	11·6	11·2	11	11·2	11	10·87	11	11·07	11
Ala	16·8	16·3	13·7	14	18·0	18	18·04	18	17·94	18
Val	7·44	7·19	8·00	8	6·15	6	6·16	6	5·98	6
Met¶	1·34	0·593	2·15	2	1·52	2	1·85	2	1·87	2
Ile†	8·40†	8·74†	7·59†	8	8·71†	9	9·07†	9	9·35†	9
Leu	17·6	17·0	16·0	16	17·3	17	16·94	17	17·19	17
Tyr	2·47	2·27	2·53	3	2·00	2	1·84*	2	1·86*	2
Phe	6·87	7·11	6·85	7	6·85	7	6·78	7	6·62	7
Lys	17·4	16·3	17·2	17	20·1	20	19·74	20	20·02	20
His	9·54	8·34	8·90	9	10·9	11	10·93	11	10·97	11
NH_3	(11·6)	(32·4)	(14·1)	(14)‖	(14·3)	(14)‖	(13·52)*‡	(14)‖	(12·78)*‡	(13)‖
Arg	3·06	3·54	3·69	4	2·83	3	2·59	3	3·00	3
Trp§	N.D.	N.D.	N.D.	—	N.D.	—	1·82§	2	1·76§	2
Total				144		149		151		151

* Values obtained by extrapolation to zero hydrolysis time.
† Isoleucine was corrected for the formation of alloisoleucine.
‡ Ammonia was corrected for the ammonia formed in the decomposition of serine and threonine.
§ Values of tryptophan represent the averages of two independent determinations.
‖ Value was not included in total.
¶ Methionine sulphone and sulphoxides were not formed and no correction was necessary.

0·065. Amino acid analysis of the phenyl isothiocyanate-treated protein in the aqueous phase showed a decrease in valine from 5·98 to 5·04mol./mol. of Mb. The ether phase from the second cyclization step contained (in mol./mol. of Mb): tyrosine, 0·672; glycine, 0·260; leucine, 0·200; serine, 0·178; aspartic acid, 0·173; alanine, 0·135. The water phase showed a decrease only in the tyrosine content from 1·90 to 1·16mol./mol. of Mb. In the third cyclization step the ether extract contained (in mol./mol. of Mb): glycine, 0·552; valine, 0·109; alanine, 0·105; leucine, 0·097; isoleucine, 0·087; glutamic acid, 0·070; aspartic acid, 0·058. This suggested to us a glycine residue in position 3. However, amino acid analysis of the protein in the aqueous phase revealed no detectable decrease in the glycine content, but, instead, a decrease in the threonine content equivalent to 1mol./mol. of Mb was obtained. The discrepancy might have been caused by the hydrolysis of some of the more labile bonds that accompany slow cyclizations. The cyclization of the *N*-terminal glycine was carried out for 6hr. Margoliash (1962) reported that it is about 80% complete in 10–12hr. On the other hand, the glycine in the ether extract of the phenylthiohydantoin amino acids could have been from the decomposition of phenylthiohydantoin tryptophan that takes place on acid hydrolysis (Fraenkel-Conrat, Harris & Levy, 1955). Also, phenylthiohydantoin threonine undergoes decomposition on acid hydrolysis, which would account for the discrepancy between the amino acid contents of the ether and water phases. The identity of the residue in position 3 therefore remains uncertain. In the fourth cyclization the ether extract contained (in mol./mol. of Mb): leucine, 0·570; glycine, 0·362; alanine, 0·192; glutamic acid, 0·139; aspartic acid, 0·131; phenylalanine, 0·061. For the protein in the

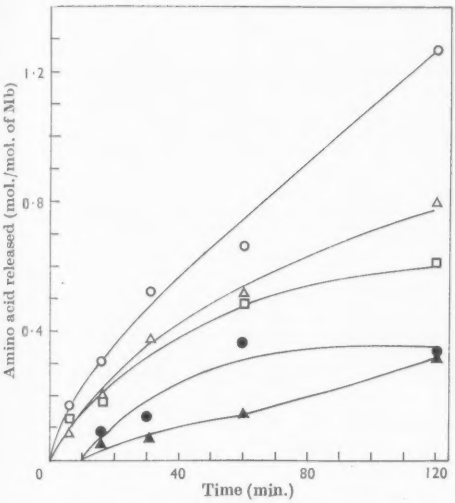

Fig. 7. Rate of release of amino acids from FMb-VII by digestion with carboxypeptidase A. ○, Serine; △, glycine; □, lysine; ●, alanine; ▲, aspartic acid. Experimental details are given in the text.

aqueous phase, the only change was observed in the leucine content, which decreased from 16·65 to 15·57 mol./mol. of Mb. The amino acid in position 4 was most probably a leucine residue. In conclusion, the *N*-terminal sequence of FMb was:

Val-Tyr-(Gly or Thr)-Leu-
1 2 3 4

Valine was also reported as the *N*-terminus of FMb by Schmid (1949).

Amino acids liberated by the action of carboxypeptidase. Fig. 7 shows the rate of release of amino acids from FMb-VII by digestion with carboxypeptidase. Only the first five amino acids are shown. These were released in the order: serine > glycine > lysine > alanine > aspartic acid. Since more than 1 mol. of serine/mol. is released, this amino acid is most probably present in more than one position in the *C*-terminal sequence. Lysyl peptide bonds are not hydrolysed by carboxypeptidase A to a significant extent. The results therefore suggest that the carboxypeptidase A preparation was appreciably contaminated with carboxypeptidase B.

DISCUSSION

The chromatographic resolution of SPMb, in its cyanmet form, into at least 12 components has been reported (Atassi, 1964*a*). These components showed a tendency towards transformation of each component into some, or most, of the others, as revealed by both chromatography and electrophoresis. The extent of conversion in a given time was dependent on the particular component. The situation with FMb, reported in the present paper, seems to be similar to that of SPMb. Also, Atassi (1964*b*) reported that the two forms of normal adult haemoglobin, A_1 and A_0 (in their met, cyanmet or oxy forms), were interconverted freely in aqueous solution. As with SPMb and adult haemoglobin, the heterogeneity of FMb does not seem to be the result of different degrees of aggregation of the protein. The existence of chromatographic components of myoglobin that possess identical amino acid compositions and different electrophoretic charges, but are interconverted in solution, might be the result of conformational changes in the protein. No explanation can be offered at the moment for the nature of these changes, which did not seem to affect the immunochemical properties of the protein. Therefore the chromatographic fractions of SPMb (Atassi, 1964*a*) and those of adult haemoglobin (Atassi, Brown & McEwan, 1965) had identical complement-fixing and antibody-precipitating efficiency when treated with antibodies to the major components of SPMb and adult haemoglobin respectively. The heterogeneities reported for some other myoglobins by various workers have been discussed by Atassi (1964*a*).

Since only very slight differences could be observed in the chromatographic and electrophoretic properties of the major components of SPMb and FMb, it is likely that these two haemoproteins are not too different in primary structure. The first evidence that they are not identical came from the infrared spectroscopic studies. This was unexpected, since proteins with the folded-chain configuration usually show almost identical spectra and this technique cannot, as a rule, be used for the identification or differentiation of individual proteins (Halford, 1957). Conformational factors, rather than alterations in primary structure, were responsible for the differences in the infrared spectra of SPMb and FMb. This was confirmed by the finding that the spectra of the globins were identical.

In addition to conformational differences that were responsible for the variations in the infrared spectra of FMb and SPMb, the two proteins probably had differences in their primary structures. These primary structural differences could not have been so drastic as to make the proteins completely dissimilar. This is revealed from the partial antigenic similarity of the two proteins. It is probable, however, that conformational differences might account for some of the lack of the antigenic efficiency of FMb when treated with the antibody to SPMb.

Table 6. *Amino acid composition of myoglobins from various species*

References: (*a*) Konosu *et al.* (1958); (*b*) Hirs & Olcott (1964); (*c*) Timmer, van der Helm & Huisman (1957); (*d*) Karadzova, Nedkov, Atanasov & Keil (1964); (*e*) Akeson & Theorell (1960); (*f*) Perkoff *et al.* (1962); (*g*) Rumen (1960); (*h*) Timmer *et al.* (1957); (*i*) this paper; (*j*) Edmundson & Hirs (1962*a*). N.D., Not determined.

Amino acid	Amino acid composition of myoglobin (residues/mol.)									
	Pisces		Mammals							
	Blue-fin tuna (*a*)	Yellow-fin tuna (*b*)	Ox* (*c*)	Dolphin (*d*)	Horse (*e*)	Human (*f*)	Seal (*g*)	Sheep* (*h*)	Finback whale (*i*)	Sperm whale (*j*)
Asp	12	12	14	12	11	12	11	12	12	8
Thr	7	7	5	5	7	4	5	5	5	5
Ser	4	5	5	5–6	5	8	7	5	5	6
Glu	11	11	17	17	19	21	16	16	17	19
Pro	6	5	4	4	4	6	4	3	4	4
Gly	14	14	12	14	16	15	12	14	11	11
Ala	21	21	16	18	16	11	14	17	18	17
Cys (half)	1	1	0	0	0	0	0	0	0	0
Val	9	8	9	6	7	8	6	9	6	8
Met	2	3	2	2	2	3	2	3	2	2
Ile	9	10	5	8	9	7	7	6	9	9
Leu	16	17	18	20	18	16	18	19	17	18
Tyr	2	2	2	2	2	2	2	2	2	3
Phe	6	6	6	8	7	7	7	9	7	6
Lys	15	15	19	21	19	19	18	19	20	19
His	5	6	13	12	11	9	12	11	11	12
Arg	2	2	3	3	2	3	5	2	3	4
Trp	1	N.D.	N.D.	2	N.D.	3	N.D.	N.D.	2	2

* Values were calculated from the authors' data (expressed as g./100g.) assuming mol.wt. 18000.

The amino acid composition of FMb is compared (in Table 6) with the compositions of some mammalian and fish myoglobins. It is apparent that FMb is very similar to SPMb in composition. This similarity might explain the partial reaction of FMb with anti-SPMb. In fact, only small differences in composition exist between the various mammalian myoglobins. The two tuna myoglobins (Table 6) have been shown to contain cysteine residues (Konosu, Hashimoto & Matsuura, 1958; Hirs & Olcott, 1964). Brown, Martinez, Johnstone & Olcott (1962) have found, in addition to the above tuna haemoproteins, that myoglobins from Albacore tuna, Shipjack tuna and Chinook salmon also contained cysteine. It would be significant to determine whether this is a general property of fish myoglobins. Table 6 shows that no mammalian myoglobin so far investigated contains cysteine. The contents of mammalian myoglobins in the basic amino acids (lysine plus histidine plus arginine) appear to be remarkably constant if expressed in 'residues per cent', i.e. (no. of residues of lysine + histidine + arginine) $\times 100$/(total no. of residues/mol.). The mean value of this content for the eight myoglobins in Table 6 is $22{\cdot}15 \pm 1{\cdot}78$ residues per cent. The basic amino acid content of the two tuna myoglobins appears to be considerably lower (blue-fin tuna, 15·38; yellow-fin tuna, 15·75 residues per cent). The two fish myoglobins also have a characteristically higher non-polar amino acid (alanine plus valine plus isoleucine plus leucine) content than the mammalian myoglobins (mammalia, $30{\cdot}6 \pm 3{\cdot}4$; blue-fin tuna, 38·5; yellow-fin tuna, 38·4 residues per cent). We have shown that constant differences in composition also exist between mammalian and fish haemoglobins. The significance of these composition characteristics has also been discussed in detail (Atassi, 1965*b*) in relation to structure, function and evolution of these haemoproteins. In conclusion, it is sufficient to point out here that FMb, with contents 22·36 residues per cent in basic amino acids and 32·9 residues per cent in the non-polar amino acids, can be classified as an average mammalian myoglobin.

We thank Dr Ray K. Brown for valuable discussions. This work was supported by the Office of Naval Research, Biochemistry Branch, Contract no. Nonr-4564(00), and by a grant (no. AM08804–01) from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, U.S. Public Health Service.

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The Inhibition of Enzymes by Beryllium

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(*Received* 1 *July* 1965)

1. The action of beryllium on the following enzymes has been examined: alkaline phosphatase (*Escherichia coli* and kidney), acid phosphatase, phosphoprotein phosphatase, apyrase (potato), adenosine triphosphatase (liver nuclei, liver mitochondria, brain microsomes), glucose 6-phosphatase, polysaccharide phosphorylases *a* and *b*, phosphoglucomutase, hexokinase, phosphoglyceromutase, ribonuclease, A-esterase (rabbit serum), cholinesterase (horse serum), chymotrypsin. Alkaline phosphatase and phosphoglucomutase are inhibited by 1μM-beryllium sulphate whereas the other enzymes are largely unaffected by 1mM-beryllium sulphate. 2. Possible mechanisms for the inhibition of phosphoglucomutase and alkaline phosphatase are discussed.

Beryllium salts are toxic to mammals and this toxicity is due to the Be^{2+} ion and not the anions of the salts (Aldridge, Barnes & Denz, 1949; Siem, 1886; Comar, 1935; Sterner & Eisenbud, 1951). After intravenous doses of beryllium salts, e.g. the sulphate, the toxic effect is due to a liver necrosis (Aldridge *et al.* 1949). However, in industry there is much evidence of chronic toxicity (Tepper, Hardy & Chamberlain, 1961) and of the development of a hypersensitive reaction to beryllium (McCord, 1951; Sterner & Eisenbud, 1951; Curtis, 1959; Waksman, 1959), though not everyone accepts this view (Hardy, 1962). In view of the suggestions that an immunological reaction is involved in the chronic human disease, it is important to know for what kind of proteins beryllium has an affinity and how it is combined with them.

It is certain that beryllium has little general affinity for proteins, e.g. those present in blood plasma (Feldman, Havill & Neuman, 1953; Reeves & Vorwald, 1961). Therefore a study of the inhibitory action of beryllium on various enzymes has been made to discover, if possible, the characteristics of those enzymes that have affinity for beryllium. Beryllium inhibits alkaline phosphatase at low concentrations (Grier, Hood & Hoagland, 1949; Aldridge, 1950; Klemperer, Miller & Hill, 1949; DuBois, Cochran & Mazur, 1949) and phosphoglucomutase at higher concentrations (Cochran, Zerivic & DuBois, 1951). The sensitivity of other hydrolases and phosphotransferases has therefore been studied. Only alkaline phosphatase and phosphoglucomutase are inhibited.

METHODS AND MATERIALS

Special chemicals. The following chemicals were obtained from the sources indicated: sodium β-glycerophosphate, dipotassium salt of glucose 1-phosphate, barium and dipotassium salts of glucose 6-phosphate, clupeine, salmine, L-cysteine hydrochloride and $BeSO_4,4H_2O$ from British Drug Houses Ltd., Poole, Dorset; glycylglycine, glycogen and yeast nucleic acid from Roche Products Ltd., Welwyn Garden City, Herts.; casein (Hammerstein) from Hopkin and Williams Ltd., Chadwell Heath, Essex; barium salt of 3-phosphoglyceric acid from Koch–Light Laboratories Ltd., Colnbrook, Bucks.; disodium salts of ATP and AMP from Sigma Chemical Co., St Louis, Mo., U.S.A.; barium salt of 2,3-diphosphoglyceric acid from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany; diethyl *p*-nitrophenyl phosphate from Albright and Wilson Ltd., Birmingham.

Enzyme preparations and determinations

Alkaline phosphatase. The enzyme was purified from bovine kidney by the method of Abul-Fadl & King (1949). The precipitate from acetone (55%, v/v) was dissolved in water to give an approx. 2% solution and dialysed free from phosphate. The activity of this preparation was 6·6μ-moles/min./mg. of protein N at 38°. The enzyme from *Escherichia coli* was also used (Worthington Biochemical Corp., Freehold, N.J., U.S.A.).

Sodium β-glycerophosphate or sodium phenyl phosphate was used as substrate and the medium, pH10·0, had the following composition: substrate (12·5mM), Na_2CO_3 (50mM), $NaHCO_3$ (50mM), magnesium acetate (25mM). In a few experiments with bovine kidney alkaline phosphatase EDTA (0·08mM) was added. It was never used with the *E. coli* phosphatase. The enzyme was usually preincubated at 38° for 5min. with the buffer containing magnesium or

beryllium or both, and the determination started by the addition of substrate. The inorganic phosphate liberated during 10min. was determined by the method of Fiske & Subbarow (1925) after precipitation of protein by $HClO_4$. Liberated phenol was determined as described by King (1951).

Acid phosphatase. Rat-liver mitochondria were isolated as previously described (Aldridge, 1957; Aldridge & Threlfall, 1961), except that the mitochondria were isolated by centrifuging at 6000*g* for 15min. The mitochondria from 8g. wet wt. of liver were suspended in 16ml. of water and dialysed against deionized water to remove sucrose and inorganic phosphate. This procedure causes the lysosomal acid phosphatase to become activated. Enzyme activity was determined as described by Berthet & de Duve (1952), with sodium β-glycerophosphate as substrate.

Phosphoprotein phosphatase. A crude enzyme preparation was obtained by homogenizing the spleens from two rats in 0·3M-sucrose by using a Potter–Elvehjem-type homogenizer with a Perspex pestle and a smooth glass tube (Aldridge, Emery & Street, 1960). The initial homogenization was made with a homogenizer with total clearance between pestle and tube of 0·02in., followed by the use of one with a clearance of 0·005in., both rotating at 1100rev./min. Debris was removed by centrifugation at 1100*g* for 10min. The supernatant and washings were dialysed against deionized water at 0° to remove inorganic phosphate. Enzyme activity was measured by the method of Paigen (1958).

Phosphoglucomutase. Enzyme was prepared from rabbit muscle by the method of Jagannathan & Luck (1949) to the stage of heat treatment at 50° at pH5·0. After cooling, the precipitate was removed and the clear supernatant was dialysed against running distilled water to remove inorganic phosphate. The solution was stored frozen. The activity of this preparation was 0·2μmole of substrate/min./mg. of protein. From the turnover number of this enzyme (Najjar, 1948) this preparation was 12% pure. Enzyme activity in the presence of cysteine was measured by the method of Najjar (1948). The concentration of reagents were as stated by Najjar (1948), with the addition of a final concentration of 5·2mM-$KHCO_3$ added as 0·4ml. of 21mM-$KHCO_3$ saturated with CO_2+N_2 (5:95). For a few experiments a crystalline preparation was used (C. F. Boehringer und Soehne G.m.b.H.).

Potato apyrase. The enzyme was prepared as described by Lee & Eiler (1951). Enzymic activity was measured in a medium of the following composition: $MgSO_4$ (1mM), disodium salt of ATP, pH7·5 (9mM), 0·4ml. of $KHCO_3$ (21mM) saturated with CO_2+N_2 (5:95); the total volume was 1·0ml. The inorganic phosphate liberated was determined by the method of Fiske & Subbarow (1925), slightly modified to prevent errors due to hydrolysis of ATP (Aldridge, 1962).

Liver-mitochondrial adenosine triphosphatase. Rat-liver mitochondria were isolated (Aldridge, 1957, 1958) and enzymic activity was measured as described by Aldridge & Stoner (1960) after activation by the addition of 30μM-2,4-dinitrophenol or by lysis in distilled water.

Brain microsomal adenosine triphosphatase. The microsomal fraction from rat brain was isolated and the adenosinetriphosphatase activity determined in the presence of sodium and potassium as described by Aldridge (1962).

Glucose 6-phosphatase. The microsomal fraction from rat liver was isolated as described above and was washed with and suspended in 0·1M-maleate buffer, pH5·4, to remove contaminating phosphorylases and phosphoglucomutase. Enzyme activity was measured as described by Swanson (1955).

Muscle phosphorylase. The enzyme was extracted from rabbit muscle and purified to the end of stage 2 as described by Cori, Illingworth & Keller (1955). The precipitated enzyme was dissolved and dialysed for 2hr. against distilled water. Enzymic activity with and without the addition of AMP was determined as described by Cori *et al.* (1955).

Phosphoglyceromutase. A partial purification was carried out from rabbit muscle by using the method of Sutherland, Posternak & Cori (1949). Enzymic activity was determined essentially by the method of Cowgill & Pizer (1956) by measuring the change in optical rotation when 3-phosphoglyceric acid is converted into 2-phosphoglyceric acid or vice versa. The medium contained: imidazole buffer, pH7·0 (2·5mM), 3-phosphoglyceric acid, pH7·0 (134mM), or 2-phosphoglyceric acid, pH7·0 (20mM), and 2,3-diphosphoglyceric acid, pH7·0 (0·02mM). Samples were taken at zero time and at 10min. and added to 1·7ml. of 25% (w/v) ammonium molybdate, and the change in optical rotation was measured with 5cm. polarimeter tubes. Phosphoglyceromutase preparations from Koch–Light Laboratories Ltd. and C. F. Boehringer und Soehne G.m.b.H. were also used.

Liver nuclear adenosine triphosphatase. Nuclei were isolated from rat liver as described by Stirpe & Aldridge (1961) and enzymic activity was determined as described by Aldridge & Stoner (1960).

A-esterase. Rabbit serum was used and was incubated with 30μM-diethyl *p*-nitrophenyl phosphate at 37° to inhibit any B-esterases present. Enzyme activity was measured by a manometric technique with phenyl acetate as substrate (Aldridge, 1953*b*).

Yeast ribonuclease. A commercial enzyme preparation (Koch–Light Laboratories Ltd.) was used and enzymic activity determined by a manometric method (Bain & Rusch, 1944). Yeast nucleic acid was purified by the method of Smith & Markham (1950).

Cholinesterase. Horse serum was used and enzymic activity determined by a manometric technique with butyrylcholine (30mM) as substrate (Aldridge, 1953*a*).

Chymotrypsin. Crystalline chymotrypsin (Armour and Co. Ltd., Eastbourne, Sussex) was used and enzymic activity measured with *N*-acetyl-L-phenylalanine ethyl ester as substrate by a manometric technique (Parks & Plaut, 1953).

Hexokinase. This enzyme was prepared from yeast by Mr V. H. Parker by a modification of the method of Berger, Slein, Colowick & Cori (1946), and activity was measured by a modification of the method of Berger *et al.* (1946). The medium contained $NaHCO_3$ (20mM), glucose (31mM), $MgCl_2$ (6·3mM), disodium ATP, pH7·4 (5mM), 1·5ml. of enzyme diluted with 1% (w/v) glucose; the total volume was 3·2ml. The solution was gassed with CO_2+N_2 (5:95) at 37° and the reaction started by the addition of the ATP and $MgCl_2$ from side arm. Readings were taken at 2min. intervals for 10min.

RESULTS

Inhibition of enzymes by beryllium. Table 1 shows the results of the treatment of a variety of enzymes with beryllium. Of these enzymes only

two are inhibited by beryllium sulphate at concentrations of 10 μM or lower. Of the eight phosphohydrolases tested only alkaline phosphatase (from both kidney and *E. coli*) is inhibited at low concentrations (1 μM or less) of beryllium sulphate, and of the phosphotransferases only phosphoglucomutase is inhibited, hexokinase and phosphoglyceromutase being unaffected by much higher concentrations. Other enzymes tested include muscle phosphorylase *a*, ribonuclease, A-esterase, cholinesterase (serum) and chymotrypsin. All are unaffected by concentrations of beryllium sulphate up to 0·4 mM.

The use of beryllium salts presents some difficulties because of a tendency to form insoluble compounds (hydroxide, phosphates etc.) or complexes (pyrophosphate, citrate etc.). We have tried therefore to check that the reagents used in the enzyme assays listed in Table 1 do not prevent inhibition of alkaline phosphatase. It was found that most of the reagents (imidazole, ATP, glycylglycine, sodium maleate, sodium acetate, albumin, 2-phosphoglyceric acid, 2,3-diphosphoglyceric acid) are unlikely to prevent inhibition by beryllium of enzymes intrinsically as sensitive as phosphoglucomutase or alkaline phosphatase. Alkaline phosphatase is inhibited by beryllium when β-glycerophosphate, glucose 1-phosphate and glucose 6-phosphate are used as substrates in bicarbonate–carbonate buffers, and phosphoglucomutase is inhibited in the presence of high concentrations of cysteine. However, there is evidence that a substrate for phosphoglyceromutase (3-phosphoglyceric acid) does form a complex with beryllium. Colour formation between aurinetricarboxylic acid and beryllium is decreased and inhibition of *E. coli* phosphatase by beryllium is less when one of the above substrates is added. *E. coli* phosphatase was assayed at pH 8·2 (by measuring *p*-nitrophenol liberation from *p*-nitrophenyl phosphate) in the presence and absence of those concentrations of 2-phosphoglyceric acid and 2,3-diphosphoglyceric acid used in the assay of phosphoglyceromutase. With both of the above conditions for the determination of enzyme activity the inhibition after preincubation of the enzyme with 1 mM-beryllium sulphate was the same. It is therefore considered unlikely that phosphoglyceromutase can be as intrinsically sensitive to beryllium as either *E. coli* phosphatase or phosphoglucomutase; preincubation of phosphoglyceromutase with 1 mM-beryllium sulphate for 5 min. at 37° leads to 15% inhibition; to produce the same inhibition of phosphoglucomutase would require only 0·4 μM-beryllium sulphate (Aldridge & Thomas, 1964).

DISCUSSION

Alkaline phosphatase from ox kidney and *E. coli* is inhibited by beryllium sulphate at micromolar concentrations. It has been known for some time

Table 1. *Effect of beryllium on various enzymes*

Beryllium sulphate was used and in each case the enzyme was preincubated with beryllium for at least 10 min. in the absence of substrate. At pH above 7 precipitates were obtained with concentrations of $BeSO_4$ of 1 mM and above. Inhibition at these concentrations may be non-specific.

Enzyme	Activated by Mg^{2+}	pH of assay	Effect of $BeSO_4$ at the concn. indicated
Alkaline phosphatase (kidney)	+	9·4	50% inhibition, 1 μM
Alkaline phosphatase (*E. coli*)	+	8·1	50% inhibition, 0·5–1 μM
Acid phosphatase	–	5·0	No inhibition, 0·6 mM
Phosphoprotein phosphatase	–	6·0	No inhibition, 0·1 mM
Apyrase (potato)	+	7·5	No inhibition, 4·0 mM
Adenosine triphosphatase (liver nuclei)	+	6·8	No inhibition, 0·5 mM; 97% inhibition, 5 mM
Adenosine triphosphatase (liver mitochondria)	+	6·8	No inhibition, 0·2 mM; 40% inhibition, 2·1 mM
Adenosine triphosphatase (brain microsomes)	+	7·4	20% inhibition, 0·64 mM
Glucose 6-phosphatase	–	6·5	No inhibition, 0·8 mM
Polysaccharide phosphorylase *a*	–	6·0	No inhibition, 0·64 mM; 91% inhibition, 6·4 mM
Phosphoglucomutase	+	7·5	50% inhibition, 5 μM
Hexokinase	+	7·4	45% inhibition, 1·5 mM; no inhibition, 0·15 mM
Phosphoglyceromutase	–	7·0	No inhibition, 2·0 mM*; 15% inhibition, 1·0 mM†
Ribonuclease	–	7·5	No inhibition, 1·0 mM
A-esterase (rabbit serum)	–	7·6	No inhibition, 1·0 mM
Cholinesterase (horse serum)	–	7·6	No inhibition, 1·0 mM
Chymotrypsin	–	7·0	10% inhibition, 1·0 mM

* 134 mM-3-Phosphoglyceric acid as substrate.
† 20 mM-2-Phosphoglyceric acid as substrate.

that mammalian alkaline phosphatase of various tissues is inhibited by beryllium [calf intestinal mucosa (Morton, 1955); rat intestine, kidney and bone (Grier *et al.* 1949); hog and rabbit kidney (Klemperer *et al.* 1949; Aldridge, 1950); rat and guinea-pig serum (Cochran *et al.* 1951; Schubert & Lindenbaum, 1954; DuBois *et al.* 1949)]. The alkaline phosphatase present in *E. coli* grown on orthophosphate-deficient media but containing β-glycerophosphate (Garen & Levinthal, 1960) is also inhibited by beryllium (Plocke, Levinthal & Vallee, 1962). Although the alkaline phosphatases from the mammalian sources listed above are activated by Mg^{2+} among other ions (Roche & Thoai, 1950; Cloetens, 1941; Clark & Porteous, 1963), there is little or no activation of the enzyme from *E. coli* by magnesium salts (Plocke & Vallee, 1962; Plocke *et al.* 1962; Garen & Levinthal, 1960).

Cochran *et al.* (1951) state that, in the absence of magnesium salts, 59 μM-beryllium chloride inhibits phosphoglucomutase by 64, 45, 93 and 48% in homogenates of rat skeletal muscle, rat liver, guinea-pig skeletal muscle and guinea-pig liver respectively. They further state that, in the presence of 1·5 mM-magnesium chloride, 0·59 mM-beryllium chloride only produces 5% inhibition. The purified phosphoglucomutase preparation from rabbit muscle is much more sensitive to inhibition by beryllium. The inhibitory process shows some rather unusual characteristics; it is competitive with respect to magnesium but is progressive, and when the inhibition is established it is no longer reversed by adding magnesium sulphate (Aldridge & Thomas, 1964). The inhibitory process is reminiscent of the activation process (Robinson & Najjar, 1961).

Of the enzymes examined in this paper (Table 1) or by other workers (Table 2) there are only two, alkaline phosphatase and phosphoglucomutase, for which there is clear evidence of a direct action of beryllium on the enzyme. For the others reported in the literature to be inhibited by higher concentrations of beryllium there is evidence that this is due to combination of beryllium with the substrate, depleting the enzyme of its usual magnesium–substrate complex [yeast hexokinase (Gutfreund & Hammond, 1963); enolase (Malmström, 1955); yeast pyrophosphatase (Bailey & Webb, 1944)]. The inhibition of phosphatidic acid phosphatase may be of the same type (Hokin, Hokin & Mathison, 1963). The only remaining report is the inhibition of amylase (McGeachin *et al.* 1962), but, in addition to the requirement for rather high concentrations of beryllium sulphate, inhibition was much less when beryllium acetate was used, making it uncertain whether the inhibition was due to Be^{2+} ion. Inhibition by beryllium sulphate at micromolar concentrations seems therefore to be confined to two enzymes. Is there, then, any common feature between alkaline phosphatase and phosphoglucomutase? There is good evidence that they both form phosphoryl-enzyme intermediates. Phosphoglucomutase is normally isolated as the phosphoryl-enzyme (Jagannathan & Luck, 1949; Najjar & Pullman, 1954; Milstein & Sanger, 1961; Sidbury & Najjar, 1957; Koshland &

Table 2. *Reported action of beryllium on enzymes and enzyme systems*

References: 1, Klemperer (1950); 2, Gutfreund & Hammond (1963); 3, Ashmore, Hastings & Nesbett (1954); 4, Axelrod, Saltman, Bandurski & Baker (1952); 5, Malmström (1955); 6, McGeachin, Pavord & Pavord (1962); 7, Bailey & Webb (1944); 8, Hurst, Little & Butler (1951); 9, Klemperer *et al.* (1949); 10, Hokin *et al.* (1963).

Enzyme	Activating ion present	Inhibition	Concn. of $BeSO_4$ (mM)	Reference
Adenosine triphosphatase (myosin)	Ca^{2+}	–	1·0	1
Hexokinase (yeast)	Mg^{2+}	+		2
Succinate-oxidase system	Al^{3+}	Activation		1
Carboxylase (yeast)	Mg^{2+}	–		1
Arginase	Mn^{2+}	–	2·6	1
Carbonic anhydrase	–	–	1·0	1
Uricase	–	–	1·0	1
Glycolysis (hexose diphosphate to lactic acid)	Mg^{2+}	–	1·0	1
Glucose 6-phosphatase	–	–	1·0	3
Phosphohexokinase (pea)	Mg^{2+}	–	5·0	4
Enolase	Mg^{2+}	+	0·5	5
Amylase	Ca^{2+}	+	0·1–1·0	6
Pyrophosphatase (yeast)	Mg^{2+}	+		7
5-Nucleotidase	Mg^{2+}	–	1·0	8
Acid phosphatase (hog kidney)	–	–	0·1	9
Phosphatidic acid phosphatase (erythrocyte membrane)	Na^{+}, Mg^{2+}	+	0·1	10

Erwin, 1957). Alkaline phosphatase from *E. coli* is phosphorylated on incubation with [^{32}P]orthophosphate in the range pH 5–6, when the enzyme is not active (Engström, 1961, 1962; Schwartz & Lipmann, 1961; Milstein, 1963; Schwartz, Crestfield & Lipmann, 1963; Schwartz, 1963); at pH10, when the enzyme is fully active, little or no labelling can be found. The rate of dephosphorylation of the phosphoryl-phosphatase under conditions when the enzyme is active has been determined and has been shown to be at least 3–4 times the maximum turnover rate (determined with *p*-nitrophenyl phosphate) (Aldridge, Barman & Gutfreund, 1964). The phosphoryl-enzyme therefore has properties that are consistent with its being an intermediate in the enzymic mechanism. For none of the enzymes studied in this paper is there certain evidence of a phosphoryl-enzyme intermediate. For some of the enzymes examined there is no evidence. For others there are either positive results against [hexokinase (Hass, Boyer & Reynard, 1961; Trayser & Colowick, 1961; Crane, 1962)] or results of kinetic measurements or exchange experiments that are consistent with the formation of a phosphoryl-enzyme intermediate. However, in most cases the authors indicate that other mechanisms are possible [myosin (Levy & Koshland, 1958; Koshland, Budenstein & Kowalsky, 1954); glucose 6-phosphatase (Hass & Byrne, 1960; Nordlie & Arion, 1964)]. Phosphorylase *a* is a phosphoprotein but does not catalyse any exchange between ^{32}P-labelled enzyme and orthophosphate (Krebs, Kent & Fischer, 1958); neither does the phosphate of AMP interchange in a reaction containing [^{32}P]-orthophosphate, glucose 1-phosphate, glycogen and phosphorylase *b* (Cohn & Cori, 1948). For this enzyme the bound phosphoryl group does not appear to be intimately involved in the catalytic process (Brown & Cori, 1961). An interesting case is phosphoglyceromutase from muscle, which might be expected to operate by a similar mechanism. Although early work seemed to indicate that a phosphoryl-enzyme intermediate was involved (Pizer, 1958, 1960; Grisolia, Joyce & Fernandez, 1961), there have always been doubts about whether this was qualitatively similar to the phosphoglucomutase in view of the different stability of the intermediate to acid and lack of exchange of the label with substrate (Pizer, 1962). Zwaig & Milstein (1963) have presented evidence that the acid-stable component of labelling (phosphorylserine) is entirely due to contamination of the enzyme preparation with phosphoglucomutase, and that the component stable under milder conditions is an enzyme–2,3-diphosphoglycerate complex. Beryllium does not inhibit this enzyme. Alkaline phosphatase and phosphoglucomutase have two things in common: a phosphoryl-enzyme intermediate step in their mechanisms, with the phosphate after acid treatment attached to serine, and sensitivity to beryllium. Enzymes, such as cholinesterase or chymotrypsin, that are phosphorylated on serine by organophosphorus compounds are not inhibited by beryllium (Table 1). It is tempting to suggest that beryllium attaches itself to alkaline phosphatase via the phosphate group; this seems unlikely since a given concentration of beryllium sulphate inhibits less when it is added to the enzyme after the substrate instead of before (W. N. Aldridge, unpublished work). It seems probable therefore that beryllium combines with the unphosphorylated enzyme in a way so that magnesium cannot compete for the unphosphorylated enzyme. Beryllium also seems to combine with phosphoglucomutase in such a way that the inhibition cannot be reversed by magnesium (Aldridge & Thomas, 1964). The common factors in the mechanism of the sensitivity of these two enzymes remain to be elucidated, but, in view of the increasing evidence for conformational changes in the protein during the catalytic process (Koshland, 1964), the possibility that beryllium either initiates unfavourable changes or prevents essential changes must be considered.

We thank Mr B. W. Street and Mr D. J. Moore for valuable technical assistance.

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The Inhibition of Phosphoglucomutase by Beryllium

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(*Received* 1 *July* 1965)

1. The inhibition of phosphoglucomutase by beryllium has been examined. 2. Inhibition by beryllium does not occur unless a complex-forming agent such as cysteine or imidazole is present. It is therefore similar to activation by magnesium. 3. The inhibition is progressive and the rate follows first-order kinetics, which may be defined by a bimolecular rate constant. 4. In the presence of magnesium the rate of inhibition is less. By using a fixed time for inhibition competition between beryllium and magnesium may be demonstrated. After inhibition has taken place, the addition of magnesium does not reverse it.

It has previously been reported that beryllium inhibits phosphoglucomutase (D-glucose 1,6-diphosphate–D-glucose 1-phosphate phosphotransferase, EC 2.7.5.1) and that this inhibition is less in the presence of magnesium (Cochran, Zerivie & DuBois, 1951). The inhibitory activity of beryllium against this enzyme has been confirmed (Thomas & Aldridge, 1966) and the present paper describes some novel features of the kinetics of the inhibitory process. A preliminary account of this work has been published (Aldridge & Thomas, 1964).

MATERIALS AND METHODS

Special chemicals. The following chemicals were obtained from the sources indicated: dipotassium glucose 1-phosphate, cysteine hydrochloride and $BeSO_4,4H_2O$ from British Drug Houses Ltd., Poole, Dorset; imidazole from Koch–Light Laboratories Ltd., Colnbrook, Bucks.; crystalline rabbit-muscle phosphoglucomutase from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany.

Determination of phosphoglucomutase. The method used was essentially that of Najjar (1948). The final composition of the medium was: cysteine (25mM), $MgSO_4$ (1·5mM), glucose 1-phosphate (1–1·8mM), pH 7·4; the total volume was 1·6–2·0ml. The progress of the reaction was followed at 30° by the determination of the acid-labile phosphorus (3min. at 100° in N-H_2SO_4) by the method of Fiske & Subbarow (1925). The reaction was zero-order until 75% of the substrate had been converted. For some experiments, and always for those experiments where the enzyme was incubated in the absence of cysteine (and was therefore largely unbuffered), a final concentration of 4·2mM-$KHCO_3$ was added as 0·4ml. of 21mM-$KHCO_3$ saturated with CO_2+N_2 (5:95). Such an addition does not influence the inhibition by beryllium in the presence of cysteine. The time allowed for activation of the enzyme by cysteine and magnesium was never less than 1min. In the experiments described below the enzyme was often preincubated with $MgSO_4$ or cysteine alone with or without $BeSO_4$. In these cases a further 1min. with both $MgSO_4$ and cysteine present was always allowed before the substrate was added. The concentrations of $BeSO_4$ stated were always those during the preincubation, which was at 30° except where otherwise stated.

Most of the experiments were carried out with a partially purified phosphoglucomutase from rabbit muscle prepared as described by Thomas & Aldridge (1966). The response of this preparation to cysteine and $BeSO_4$ was identical with that of the crystalline preparation.

RESULTS

Complex-forming agents and inhibition by beryllium. Phosphoglucomutase requires magnesium, a complex-forming agent and glucose 1,6-diphosphate as coenzyme for maximal activity (Najjar, 1948; Robinson & Najjar, 1961; Sutherland, 1949; Cori, Colowick & Cori, 1938; Leloir, Trucco, Cardini, Paladini & Caputto, 1948; Cardini, Paladini, Caputto, Leloir & Trucco, 1949; Milstein, 1961*b*). Since the enzyme as normally isolated is in the phosphoryl-enzyme form (Jagannathan & Luck, 1949; Najjar & Pullman, 1954), no glucose 1,6-diphosphate, other than that in the glucose 1-phosphate, has been added. For most of the experiments described below cysteine has been used as the complex-forming agent because with our enzyme preparation this has given a higher activity than imidazole. In the absence of magnesium or cysteine or both the enzyme has no activity. Although Robinson & Najjar (1961) demonstrated that the activation of phosphoglucomutase by imidazole and magnesium was a time-dependent process and for full activation several minutes were required, we have been unable to demonstrate such a time-dependent activation with cysteine

and magnesium. Full activity has always been obtained when cysteine and magnesium are present at 30°; even with lower concentrations of cysteine or at 0° maximal activity was obtained with no preincubation. We conclude from these observations that cysteine is a more efficient activator than imidazole and the activation process is rapid. Since the concentration of cysteine in tissues is much less than that of glutathione (Bhattacharya, Robson & Stewart, 1955; Malathi, Seshadri Sastry & Ganguly, 1961; Thomson & Martin, 1959), glutathione has been tested as an activator of phosphoglucomutase (cf. Lehmann, 1939). In the presence of glutathione an activity only 60% of that with cysteine can be obtained; the concentration for half-maximal activation is approx. 0·5mM for each compound.

In early experiments with phosphoglucomutase and beryllium it was found that little or no inhibition was obtained if cysteine was not present during the incubation with beryllium (Table 1). Preincubation in the absence of cysteine and magnesium leads to only 7% inhibition, whereas in the presence of 25mM-cysteine 80% inhibition is obtained. Beryllium also inhibits with imidazole in the place of cysteine. If magnesium sulphate (1·5mM) is present with cysteine during the preincubation a 15-fold increase in the concentration of beryllium sulphate is required to give the same degree of inhibition.

Competition between beryllium and magnesium. The following reactions take place in the activation by magnesium and inhibition by beryllium of phosphoglucomutase:

$$E + Mg \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E\text{–}Mg \xrightarrow{+S} E\text{–}Mg\text{–}S$$

$$E + Be \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} E\text{–}Be$$

$$E\text{–}Mg\text{–}S \xrightarrow{k_3} E\text{–}Mg + \text{Products}$$

By using the steady-state kinetic analysis outlined in Dixon & Webb (1964, p. 429), with the assumption that the activation by magnesium and the reaction of the enzyme with the substrate are independent of one another, it can be shown that:

$$v = \frac{V_{Mg}}{1 + \frac{K_{Mg}}{[Mg]}}$$

where v is the velocity of reaction, V_{Mg} is the maximum velocity of reaction at a particular substrate concentration in the presence of magnesium and K_{Mg}, i.e. k_{-1}/k_{+1}, is the dissociation constant of the enzyme–magnesium complex. In the presence of another metal, I, competing with magnesium but forming an inactive enzyme complex:

$$v = \frac{V_{Mg}}{1 + \frac{K_{Mg}}{[Mg]}(1 + A[I])} \qquad (1)$$

where the combination of enzyme with metal is reversible A equals k_{+2}/k_{-2}, i.e. $1/K_i$, the reciprocal of the dissociation constant of enzyme–inhibitor complex. As shown below, the reaction of phosphoglucomutase with beryllium is progressive (i.e. k_{-2} tends towards 0) and is described by a velocity constant rather than an equilibrium constant. In these circumstances A equals k_2, the bimolecular rate constant for the reaction of enzyme with beryllium. As shown in Fig. 1, a graph of $1/v$ against $1/[Mg]$ indicates that competition between magnesium and beryllium takes place. However, as pointed out by Wilkinson (1961), from a statistical point of view, calculation of the constants for the reactions involved (K_{Mg}, V_{Mg} and k_{+2}) are better derived from the regression lines relating $[Mg]/v$ and $[Mg]$. The results of these calculations are given in Table 2, the concentration of magnesium sulphate giving half maximum activity being 75μM and the bimolecular rate constant for the reaction of enzyme with beryllium is $6{\cdot}1 \times 10^4$ l.mole^{-1}min.$^{-1}$. A value for K_{Mg} of approx. 50μM has been obtained by Milstein (1961*a*).

Table 1. *Inhibition of phosphoglucomutase by beryllium*

The medium before the addition of glucose 1-phosphate contained $MgSO_4$ (1·5mM), cysteine (25mM), $KHCO_3$ saturated with $CO_2 + N_2$ (5:95) (see the Materials and Methods section). The pH was 7·4 and the incubation with or without $BeSO_4$ was at 30° for 10min. Controls were carried out in the absence of $BeSO_4$ and enzyme activity was the same for all three conditions of incubation.

Incubation conditions	Activation for 1min.	Inhibition (%)
Enzyme + $BeSO_4$ (2·7μM)	$MgSO_4$ + cysteine	7
Enzyme + cysteine + $BeSO_4$ (2·7μM)	$MgSO_4$	80
Enzyme + $MgSO_4$ + cysteine + $BeSO_4$ (40μM)	—	80

Progressive inhibition. When phosphoglucomutase is incubated with beryllium sulphate and cysteine for various times, the inhibition increases with time and shows the characteristics of a first-order reaction (Fig. 1). The bimolecular rate constants derived from these results are $7{\cdot}2 \times 10^4$, $6{\cdot}1 \times 10^4$ and $8{\cdot}4 \times 10^4$ (mean $7{\cdot}2 \pm 1{\cdot}1 \times 10^4$) l.mole^{-1}min.$^{-1}$.

In other experiments a similar rate constant was

obtained with a crystalline enzyme preparation. The calculation of such rate constants is only valid if the concentration of one component (beryllium) of the system is effectively constant. Calculation from a molecular activity of 12400 mole^{-1} min.$^{-1}$ of enzyme (Najjar, 1962) indicates an enzyme concentration of 0·013 μM, at least 200–300 times less than the concentration of beryllium sulphate.

Inhibition of enzymes by metal ions has usually been described by an equilibrium process (Dixon & Webb, 1964, p. 346). In view of the lack of equilibrium with beryllium and phosphoglucomutase, the effect of the other constituents of the medium on the inhibitory process was determined. Milstein (1961*b*) has shown that the activation of phosphoglucomutase is a complex process and is partly due to removal of heavy-metal impurities. It was therefor essential to examine the influence of cysteine added at various times. If the enzyme is incubated with cysteine before a further incubation with beryllium, the progressive inhibition still occurs (Fig. 2); if the enzyme is incubated with beryllium in the absence of cysteine, little or no inhibition occurs. In Figs. 1, 2 and 3 the lines sometimes do not pass through 2·0 (log100%). This has been found to be somewhat variable between experiments; we have no explanation for it.

Since it has been shown above that the inhibition by beryllium is competitive with respect to magnesium, the effect of magnesium on the rate of

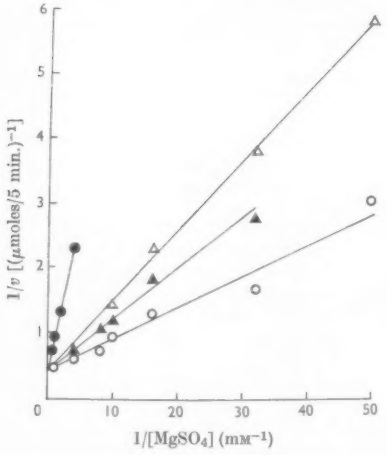

Fig. 1. Competition between beryllium and magnesium. The conditions are as given in Table 2. Concn. of $BeSO_4$: ○, none; ▲, 1·0 μM; △, 3·2 μM; ●, 16 μM. Owing to overlapping of points not all the results used for the calculations in Table 2 are included in this Figure.

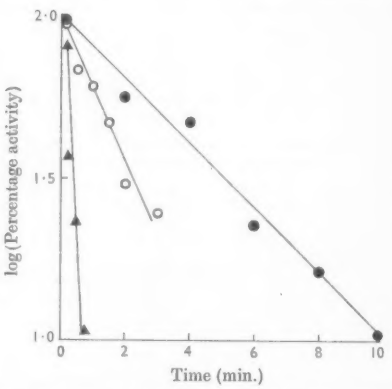

Fig. 2. Progressive inhibition of phosphoglucomutase by beryllium. The enzyme was incubated with $BeSO_4$ in the presence of cysteine (31·2 mM) for 0–10 min. (which is the time plotted); $MgSO_4$ was added and, after a further 1 min., 2 μmoles of glucose 1-phosphate. The unchanged substrate was determined at 16 min. Concn. of $BeSO_4$: ●, 3·1 μM; ○, 8·3 μM; ▲, 31·3 μM.

Table 2. *Competition between beryllium and magnesium*

The enzyme was added to the medium containing $BeSO_4$, $MgSO_4$ and cysteine. The mixture was incubated for 10 min. at 30° before adding glucose 1-phosphate (1·8 mM, total vol. 1·6 ml.). The concentration of $MgSO_4$ was in the range 0·02–2·5 mM. The values were calculated from regression lines relating [Mg]/v and [Mg], where v is the velocity of the reaction in μmoles of substrate used in 10 min. (see the text for explanation of constants).

Concn. of $BeSO_4$ (μM)	No. of observations	V_{Mg} (μmoles of substrate/5 min.)		Concn. of $MgSO_4$ giving $V_{Mg}/2$ (μM)	$10^{-4}k_{+2}$ (l. mole^{-1} min.$^{-1}$)	
0·0	15	1·96		75·5	—	
0·64	8	2·10		105	4·7	
1·0	7	1·88	Mean	130	6·0	Mean
3·2	8	2·19	1·95	219	7·3	6·1 ± 1·1
16	6	1·62		627	6·3	

inhibition has been examined. Again no inhibition is obtained if cysteine is omitted from the medium and the enzyme incubated with beryllium in the presence of magnesium (Fig. 3). If the enzyme is preincubated with cysteine or (cysteine plus magnesium) before beryllium the progressive inhibition still occurs. The rate of inhibition in the presence of 1·8 mM-magnesium sulphate and 40 μM-beryllium sulphate is approximately the same as that with 2·7 μM-beryllium sulphate in the absence of added magnesium (bimolecular rate constant $3{\cdot}2 \times 10^3$ l.mole^{-1}min.$^{-1}$ in the presence of 1·8 mM-magnesium sulphate). Magnesium therefore slows down the rate of progressive inhibition. However, if the enzyme is inhibited by incubation with beryllium sulphate (2·7 μM) and cysteine and then magnesium sulphate are added, no further inhibition occurs. This agrees with the slight inhibition expected by 2·7 μM-beryllium sulphate and 1·8 mM-magnesium sulphate (calculated from a rate constant of $3{\cdot}2 \times 10^3$ l.mole^{-1}min.$^{-1}$ this is only 8%). More important is the observation that the enzyme is not reactivated by the addition of magnesium sulphate.

From a few experiments at different temperatures (10°, 20°, 30°, 37°) the apparent energy of activation of the progressive inhibition by beryllium is 13300 ± 3200 (4) cal./mole. This is similar to the value of 14200 ± 780 (8) cal./mole for the enzymic process.

DISCUSSION

There are therefore two phenomena to be discussed. First, inhibition of phosphoglucomutase after incubation with beryllium requires the presence of a complex-forming agent (cysteine and imidazole); secondly, in the presence of a complex-forming agent the inhibition is progressive, follows first-order kinetics and shows the characteristics of a bimolecular reaction with one component in excess.

Phosphoglucomutase requires for activation both magnesium and a complex-forming agent. It seems likely that the main function of the complex-forming agent (cysteine) is to remove heavy-metal inhibitors from the enzyme (Milstein, 1961*a*,*b*) so that magnesium can gain access. It is therefore probable that the requirement for cysteine to obtain inhibition by beryllium (Table 1 and Figs. 3 and 4) is the same. The inhibition by beryllium increases with time and it is important to know that this apparent slow inhibition is not due to a slow removal

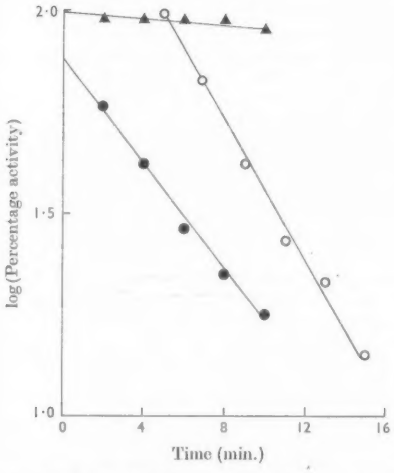

Fig. 3. Progressive inhibition of phosphoglucomutase by beryllium. The conditions before the addition of substrate were: ●, $BeSO_4$ (2·7 μM) plus cysteine (0–10 min.), $MgSO_4$ (10–11 min.); ○, cysteine (0–5 min.), $BeSO_4$ (2·7 μM, 5–15 min.), $MgSO_4$ (15–16 min.); ▲, $BeSO_4$ (2·7 μM, 0–10 min.), $MgSO_4$ plus cysteine (10–11 min.). During the above incubations the medium contained $MgSO_4$ (1·8 mM) and cysteine (31·2 mM). Enzyme activity was obtained by the addition of glucose 1-phosphate (2 μmoles) and the residual substrate determined after 5 min. Parallel controls were carried out in the absence of $BeSO_4$ for all times and conditions of incubation. In no instance did the activity of the enzyme change during the experiment. For the purpose of plotting the results the 1 min. incubations with $MgSO_4$ or cysteine or both to activate the enzyme have been ignored.

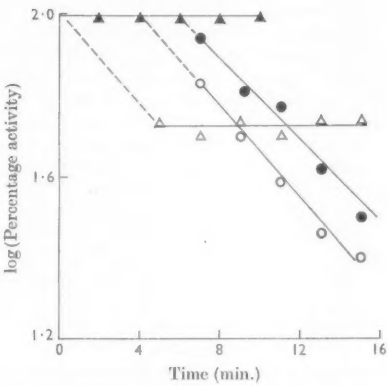

Fig. 4. Progressive inhibition of phosphoglucomutase by beryllium. The conditions before the addition of substrate were: ▲, $BeSO_4$ (40 μM) plus $MgSO_4$ (0–10 min.), cysteine (10–11 min.); ●, cysteine (0–5 min.), $BeSO_4$ (40 μM) plus $MgSO_4$ (5–15 min.); ○, cysteine plus $MgSO_4$ (0–5 min.), $BeSO_4$ (40 μM, 5–15 min.); △, $BeSO_4$ (2·7 μM) plus cysteine (0–5 min.), $MgSO_4$ (5–15 min.). Other conditions and controls are as stated in Fig. 3.

of heavy metals from the enzyme. This cannot be so, since the activation by cysteine is rapid (cf. Table 1) and in other experiments has been shown to be complete in 15sec. at 0°. Further, incubation of the enzyme with cysteine for 5min. at 30° (Figs. 3 and 4), whether with or without magnesium, followed by the addition of beryllium sulphate, does not lead to an immediate rapid inhibition but to the progressive inhibition obtained without a preliminary incubation with cysteine. Therefore the progressive inhibition is due to a reaction between the active enzyme and beryllium and is not due to a progressive reaction induced by the other reagents present.

It seems probable that the initial attachment of beryllium to the enzyme is in principle the same process as the attachment of magnesium during the activation process; this conclusion is reinforced by the demonstration of competition between magnesium and beryllium. However, such a similarity between activation and inhibition gives no clue of the processes involved at a molecular level. It is unlikely that the processes of activation by magnesium and the progressive inhibition by beryllium are formally the same. The kinetics of the inhibitory process are identical with those of the inhibition of esterases by organophosphorus compounds (Aldridge, 1956). By analogy with the reaction of enzyme with substrate it might be expected that there would be an intermediate 'esterase–organophosphate' complex before the formation of the phosphorylated enzyme. There is no direct evidence for such a complex with erythrocyte cholinesterase (Aldridge & Davison, 1952), but for human serum cholinesterase and one organophosphorus compound an 'inhibitor–enzyme intermediate' has been claimed to have been detected (Main, 1964). The inhibition of phosphoglucomutase by beryllium is analogous and there is no direct evidence of a two-or-more-stage process. For inhibition of esterases by organophosphorus compounds it is as though collision of inhibitor and enzyme in the right way leads directly to reaction to give a phosphorylated enzyme; it is possible that the inhibition of phosphoglucomutase by beryllium is similar and that the presence of magnesium merely lowers the probability of a 'fruitful' collision. Although the breakage and formation of the covalent bonds involved in the phosphorylation of esterases is compatible with an apparent energy of activation of 10–11kcal./mole (Aldridge, 1953), a value of 13–14kcal./mole for the inhibition of phosphoglucomutase by beryllium is difficult to reconcile with a simple ionic interaction of enzyme and beryllium. If no other changes occur in the protein, then the beryllium must be attached by a covalent bond to the protein. There is evidence that beryllium can behave chemically in a somewhat anomalous manner (Pauling, 1948; Van Uitert, Fernelius & Douglas, 1953); it is therefore at present not possible to eliminate a combination of beryllium with enzyme by covalent bonds. However, temperature-dependent changes in the protein initiated by the primary attachment of beryllium (instead of magnesium), leading to an inactive enzyme, must be considered. Theoretically this change could be catalytic, for we have no conclusive evidence that beryllium is attached to the inhibited enzyme.

We thank Mr B. W. Street and Mr D. J. Moore for valuable technical assistance.

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Conformational Changes and the Regulation of Glutamate-Dehydrogenase Activity

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(*Received* 31 *May* 1965)

1. The effect of NADH and the non-competitive inhibitor GTP on the optical-rotatory-dispersion properties of glutamate dehydrogenase has been studied. 2. Analysis of the data in terms of the a_0 and b_0 parameters of the Moffitt–Yang equation indicates that a conformational change is induced either by NADH or by GTP in the presence of small amounts of NADH. 3. Sedimentation measurements under comparable conditions showed that the enzyme reversibly dissociates into sub-units but that this dissociation is only secondary to the conformational changes. 4. Fluorescence measurements showed that the binding constant of NADH and the number of binding sites on the enzyme increased in the presence of GTP. 5. This is confirmed by studies of fluorescence polarization, which in addition showed that the movement of NADH on the enzyme surface is more restricted in the presence of GTP. 6. The relation of these results to possible regulatory mechanisms is discussed.

The activity of ox-liver glutamate dehydrogenase [L-glutamate–NADP oxidoreductase (deaminating), EC 1.4.1.3] is inhibited by NADH, GTP (Frieden, 1963) and a variety of other substances such as steroid hormones (Yielding & Tomkins, 1960) and phenanthroline (Yielding & Tomkins, 1962). The inhibition by GTP was shown to be of the non-competitive type (Frieden, 1963) and it was therefore suggested that this may be due to some conformational change in the protein. NADH has also been shown to be bound at sites other than the active centre with similar effects. Frieden argued (Frieden, 1963, and references therein), mainly from fluorescence measurements, that such conformational changes do occur, whereas Tomkins and his co-workers maintained that the changes in activity brought about by small molecules are associated with the dissociation of the enzyme into sub-units (Yielding, Tomkins, Bitensky & Talal, 1964). More recently Tomkins and co-workers have accepted the view that regulation of glutamate-dehydrogenase activity is achieved by inducing conformational changes in the enzyme and that dissociation is only a secondary consequence of these changes (Bitensky, Yielding & Tomkins, 1965*a*,*b*).

Information about the secondary and tertiary structure of proteins and polypeptides in solution may be gained from measurements of the wavelength-dependence of optical rotation (optical rotatory dispersion) (Urnes & Doty, 1961). The present paper reports our optical-rotatory-dispersion studies on glutamate dehydrogenase in the absence and presence of coenzyme and the non-competitive inhibitor GTP, and their correlation with fluorescence, polarization of fluorescence and sedimentation studies. A brief account of this work has already appeared (Bayley & Radda, 1965). A similar paper dealing with the optical rotatory dispersion of this system has been published by Magar (1965).

MATERIALS

Glutamate dehydrogenase was obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany, as an ammonium sulphate suspension or a 50% glycerol solution. The latter was employed for most experiments, but there was no essential difference between the two preparations. The enzyme was freed from glycerol or ammonium sulphate by gel filtration on a Sephadex G-25 column at 0° by using the appropriate buffer solution. Experiments were carried out in 0·1M-phosphate buffer, pH 7·7, unless otherwise stated. The protein concentration after gel filtration was determined from the extinction at 280 mμ, by using the published value 0·97 for $E^{0.1\%}_{1cm.}$ (Olson & Anfinsen, 1952). The homogeneity of fully active enzyme preparation from C. F. Boehringer und Soehne G.m.b.H. has been demonstrated by several workers (Sund, 1961; Frieden, 1963). The different batches used by us were also fully active and homogeneous in the ultracentrifuge.

NAD^+ and NADH were purchased from C. F. Boehringer

und Soehne G.m.b.H., GTP from Sigma Chemical Co., St Louis, Mo., U.S.A., and L-glutamic acid and AnalaR EDTA from British Drug Houses Ltd., Poole, Dorset. Butan-2-ol was freshly distilled before use (b.p. 99·5–100·5°).

METHODS

Optical rotatory dispersion was measured with a Bendix–Ericsson automatic recording spectropolarimeter (model Polarmatic 62). Measurements were performed at 25° with fixed constant slit widths (0·5mm. at the entrance slit and 0·4mm. at the exit). The instrument was frequently calibrated with a standard sucrose solution. The extinction was kept below 0·8 to eliminate possible 'spurious' effects due to scattered light, although the instrument could measure rotations fairly accurately even when the extinction was as high as 1·5. Cylindrical silica cells of 1·00cm. path length were used.

Fluorescence measurements were done on two instruments. The Zeiss spectrophotofluorimeter and the Aminco–Bowman instruments gave essentially identical results. The slit widths used were never more than 0·5mm. on the Zeiss instrument. The combination of 4mm. and 5mm. diaphrams was used on the Aminco–Bowman instrument.

Polarization of fluorescence was initially measured on the Aminco–Bowman instrument equipped with two Glan–Thompson prisms. Corrections for the inherent polarization in the monochromators was carried out by the method described by Weill & Calvin (1963). The t_2 value defined by Weill & Calvin (1963) was not more than 1·2. Some of the measurements were later repeated on the Zeiss spectrophotofluorimeter modified to accommodate two Foucault prisms of 12mm. aperture (the prisms were supplied by Bellingham and Stanley Ltd., London). The results on this instrument agreed to within 5% of those obtained on the Aminco–Bowman instrument. The corrections for polarization on the monochromators were considerably less (t_2 not more than 1·05) on the Zeiss instrument than on the Aminco–Bowman instrument, presumably since the prism-type monochromators in the former produce less polarization of the incident beam than the grating-type monochromators of the latter instrument. By convention 'vertical' polarization is defined as being perpendicular to the plane of propagation of light through the instrument. This plane is horizontal in the Aminco–Bowman instrument and vertical in the Zeiss instrument. In both the angle of observation is 90° to the incident direction. In the Aminco–Bowman instrument fluorescence is observed through the side of the cell; in the Zeiss instrument it is viewed through the bottom.

Sedimentation coefficients were determined by using a Spinco model E analytical ultracentrifuge with schlieren optics.

Enzyme reaction rates were measured as described by Hellerman, Schellenberg & Reiss (1958). The activity of the enzyme preparations was frequently determined after the physical measurements, particularly after sedimentation studies. In most cases there was no difference in the activities before and after the runs. In a few instances we found that denaturation had occurred and these results were rejected. Some inactivation occurred in the spectrophotofluorimeter if the sample was left in the light beam (cf. Chen, 1964) for ½hr. or longer. This would normally be accompanied by a fall in the fluorescence intensity of the coenzymes. Measurements were therefore carried out with minimum time of illumination of the sample in the incident beam.

RESULTS

Optical rotatory dispersion. Optical-rotatory-dispersion curves were recorded between 550 and 260mμ. The initial measurements were done in 20mM-tris–hydrochloric acid buffer, pH7·7, containing potassium chloride (0·1M). Although reasonable curves could be obtained, the solution tended to become cloudy during the measurements owing to denaturation. The solutions were found to be considerably more stable in 0·1M-phosphate buffer, pH7·7, and all later measurements were done in phosphate buffers. Generally, the enzyme solutions gave rise to some light-scattering at concentrations above 3mg./ml. The most convenient concentration region for recording the optical rotations was between 0·8 and 2mg. of glutamate dehydrogenase/ml. Fig. 1 shows the optical-rotatory-dispersion curves for the enzyme in the absence and presence of 0·1mM-NADH and in the presence of 6M-urea. The results are in agreement with Jirgensons's (1961) findings that glutamate dehydrogenase has a positive rotation in the visible region. The addition of NADH, or GTP in the presence of NADH, causes a negative shift in the rotation of the molecule. Denaturation by urea results in a further shift in the negative direction. In contrast with liver alcohol dehydrogenase (Ulmer & Vallee, 1961), binding of NADH to the protein does not produce anomalous dispersion in the region of NADH absorption.

The rotatory-dispersion curves can be analysed in terms of the Moffitt & Yang (1956) phenomenological equation:

$$[m'] = \frac{a_0\lambda_0^2}{\lambda^2-\lambda_0^2} + \frac{b_0\lambda_0^4}{(\lambda^2-\lambda_0^2)^2}$$

In this $[m']$ is the reduced mean residue rotation at

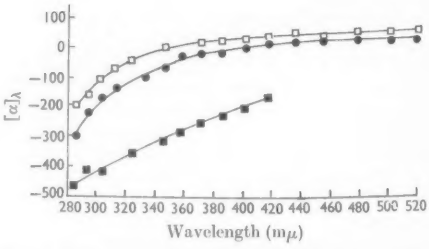

Fig. 1. Optical-rotatory-dispersion curves: □, apoenzyme; ●, apoenzyme+NADH (95μM); ■, apoenzyme in 6M-urea.

wavelength λ and is calculated from the measured specific rotation by the equation:

$$[m'] = \left(\frac{3}{n^2+2}\right) \times \frac{\text{MRW}}{100} \times [\alpha]_\lambda$$

In this equation n is the refractive index of the solvent (taken to be that of water) and MRW the mean residue weight of the protein, for which we used the value 115, commonly employed for proteins (Urnes & Doty, 1961). No correction was made for the dispersion of n.

In the calculations of the Moffitt parameters the experimental value 212 was used for the value of the constant λ_0 (Moffitt & Yang, 1956). A test for the correctness of this value lies in the good linearity of the Moffitt plots (Fig. 2). The parameters a_0 and b_0 can be calculated from the intercept on the ordinate and the slope of the lines respectively of the plots shown in Fig. 2. The results of the measurements under a variety of conditions are set out in Table 1, which also contains the results of the sedimentation studies made under conditions comparable with those used in the optical-rotatory-dispersion measurements.

Fluorescence measurements. The emission spectra of 50μM-NADH alone and in the presence of glutamate dehydrogenase (1mg./ml.) and glutamate dehydrogenase (1mg./ml.) with GTP (1mM) are recorded in Fig. 3. Light at 340mμ was used for excitation. The increase in intensity when NADH is bound to enzyme has been noted by Schwert & Winer (1958). It has also been observed that the addition of GTP to the system causes a further increase in the intensity of the emitted light, although there is disagreement about the reasons for it. According to Tomkins, Yielding & Curran (1962), the further enhancement of NADPH fluorescence is due to an increase in the number of binding sites on the enzyme as a result of dissociation; however, Frieden (1963) showed from quenching experiments that it is a result of a decrease in NADPH–enzyme dissociation constant, which gives rise to binding of more coenzyme at low enzyme concentrations. We have approached the problem from two directions. When a given

Table 1. *Moffitt parameters and sedimentation coefficients for glutamate dehydrogenase*

The enzyme concentration was 2mg./ml. The rotations reported by Bayley & Radda (1965) were expressed as those in water. The present data are corrected for the refractive index of the solvent.

Concn. of NADH (μM)	Concn. of GTP (mM)	b_0	a_0	$S_{20,w}$ (S)
0	0	−190	104	21·3
16	0	−220	104	21·3
26	0	−200	102	21·5
95	0	−250	112	23·5
133	0	−250	118	23·5
190	0	−260	66	—
200	0	—		13·0
95	0	−210	100	21·0
	(+0·25mM-EDTA)			
0	1	−210	120	21·0
21	1	−250	130	21·0 and 11
32	1	−260	110	12·0
86	1	−270	50	12·0
133	1	−260	35	12·0
16	0·1	−200	95	—
15	0·6	−230	115	20 (small peak at 11·5)
30	0·6	−250	90	11·8 (small peak at 15·7)
Apoenzyme in buffer +10% (v/v) butan-2-ol		−190	35	10·5

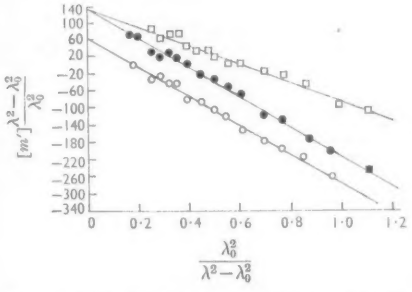

Fig. 2. Moffitt–Yang plots for glutamate dehydrogenase: □, apoenzyme; ●, apoenzyme+NADH (95μM); ○, apoenzyme+NADH (86μM)+GTP (1mM).

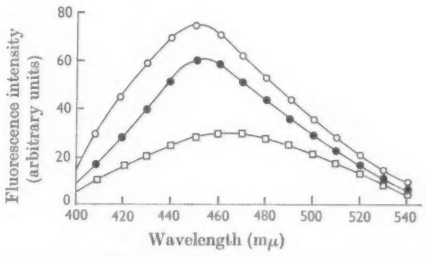

Fig. 3. Fluorescence spectra of NADH in free and bound form: □, NADH (50μM); ●, NADH (50μM)+glutamate dehydrogenase (1mg./ml.); ○, NADH (50μM)+glutamate dehydrogenase (1mg./ml.)+GTP (1mM).

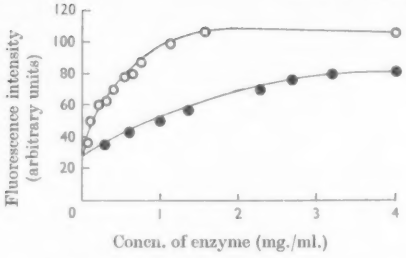

Fig. 4. Increase in fluorescence intensity of 15μM-NADH on titration with apoprotein in the absence (●) and presence (○) of GTP (1mM).

amount of NADH is titrated with increasing concentrations of protein the curves approach a limiting value that is characteristic of the completely bound coenzyme (Fig. 4). The limiting fluorescence values for the bound coenzyme depend on the presence or absence of GTP, indicating an increase in the binding constant (see the Discussion section). The second method involves titrating a constant amount of protein with increasing concentrations of the coenzyme (Fig. 5). This is limited to concentration ranges of the coenzyme where the absorption of light at 340mμ is not too great. From these curves the increase in fluorescence on binding can be derived from the intercept of the initial and final slopes of the curve and by comparing this value for the fluorescence with that of the free NADH at the same concentration. The ratios of intensities of bound to free coenzyme are 2·8 and 3·8 in the absence and presence of GTP respectively. These values agree well with the values 2·9 and 3·9 that can be derived from the first method in Fig. 4. From the limiting values of fluorescence for the bound coenzyme the fraction of NADH bound can be calculated and the data in Fig. 5 can be analysed by the method described by Scatchard, Coleman & Shen (1957). $\bar{\nu}$, i.e. mol. of NADH bound/mol. of enzyme, is calculated and $\bar{\nu}/C$ (where C is the concentration of free NADH) is plotted against $\bar{\nu}$. The slope of the line then is $-K_{assoc.}$ (the association constant for the binding) and the intercept on the abscissa gives the number of binding sites/mol. These results are summarized in Table 2.

Polarization of fluorescence. The change in the fluorescence polarization of NADH as a result of complex-formation can again be measured by the two different methods described for fluorescence above. These results are shown in Figs. 6 and 7. The difference in the polarization at the limiting value in Fig. 6 is experimentally significant and the experimental points are derived from a large

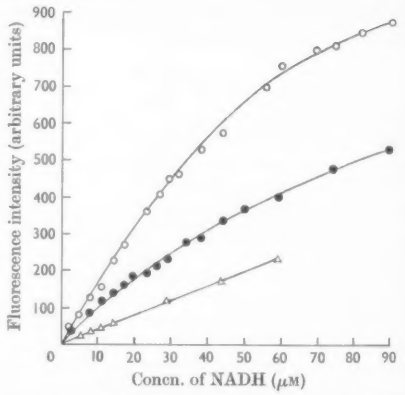

Fig. 5. Changes in fluorescence intensity on titration of glutamate dehydrogenase (1·7mg./ml.) with NADH: △, NADH alone; ●, NADH+enzyme; ○, NADH+enzyme +GTP (1mM).

Table 2. *Binding constants and number of sites for NADH on glutamate dehydrogenase*

	From fluorescence		From polarization of fluorescence	
	$10^6K_{assoc.}$ (M^{-1})	No. of sites	$10^6K_{assoc.}$ (M^{-1})	No. of sites
Without GTP	0·5	21	0·4	19
With 1mM-GTP	0·8	25	0·8	25

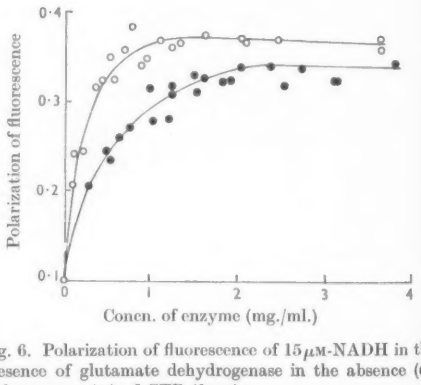

Fig. 6. Polarization of fluorescence of 15μM-NADH in the presence of glutamate dehydrogenase in the absence (●) and presence (○) of GTP (1mM).

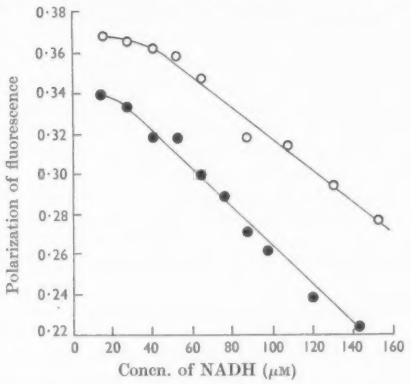

Fig. 7. Polarization of fluorescence on titration of glutamate dehydrogenase (2mg./ml.) with NADH: ●, NADH+enzyme; ○, NADH+enzyme+GTP (1mM).

number of measurements. The polarization spectrum of bound NADH shows a wavelength-independence of the exciting light throughout the 340mμ absorption band of the coenzyme.

The polarization of NADH fluorescence was measured with polarized incident light. The polarization is then given by the relation:

$$p = \frac{V_v - H_v}{V_v + H_v}$$

where the capital letter refers to the direction of the emitted radiation and the subscript to that of the incident light, V meaning vertical polarization and H horizontal polarization. The measured polarization for the NADH–glutamate-dehydrogenase system is the sum of the contributions by free and bound forms of the coenzyme; α, the fraction of coenzyme bound to the protein, is not a direct function of p (the value of polarization), however, since the total intensity of the V_v and H_v components also depends on the relative specific fluorescence intensities of the free and bound forms of the coenzyme. If I_v (free) and I_v (bound) represent the V_v components of the specific fluorescence intensities of the free and bound coenzyme respectively, and I_h (free) and I_h (bound) the H_v components, then the polarization is related to α as follows:

$$p = \frac{[(1-\alpha)\,I_v(\text{free}) + \alpha I_v(\text{bound})]}{[(1-\alpha)\,I_v(\text{free}) + \alpha I_v(\text{bound})]} \quad \frac{-[(1-\alpha)\,I_h(\text{free}) + \alpha I_h(\text{bound})]}{+[(1-\alpha)\,I_h(\text{free}) + \alpha I_h(\text{bound})]}$$

The four specific intensity components can be obtained experimentally during the measurements by comparing I_v and I_h components in the limiting case at high enzyme concentration (all coenzyme bound) and NADH without the protein present (NADH free), i.e. the components used in deriving Fig. 6. Then α can be calculated for each point in Fig. 7, and the data analysed in a manner similar to that described for the fluorescence measurements above. The binding constants and the number of binding sites calculated this way agree well with those derived from unpolarized fluorescence measurements (see Table 2).

DISCUSSION

The importance of conformational changes in the regulation of enzymic activity has been discussed in detail by Monod, Changeux & Jacob (1963). The term 'allosteric effect' defines a phenomenon in which a conformational change is induced in the enzyme (which then may lead to either activation or inhibition) by a small molecule bound at a site other than the active centre. Glutamate dehydrogenase has been assumed to be an enzyme in this class (Monod *et al.* 1963). GTP is a non-competitive inhibitor of glutamate dehydrogenase (Frieden, 1963) and both NADH and GTP are bound at a second site. They are also known to cause dissociation of the enzyme into sub-units, but it is thought that they may act differently in that the effect of NADH alone can be reversed by substances that chelate Zn^{2+} (e.g. EDTA), whereas GTP requires NADH and does not require Zn^{2+} to be effective.

The results of optical-rotatory-dispersion measurements (see Table 1) combined with the sedimentation data clearly show that NADH at concentrations higher than 0·1mM induces some change in the protein that is reflected in the b_0 value of the Moffitt equation, yet does not affect the a_0 parameter or the sedimentation coefficient, $S_{20,w}$, below 0·2mM concentration. This effect can be reversed by EDTA. Since b_0 is related to the effective helical content of proteins we may conclude that changes that affect it are due to an alteration in the secondary structure of the protein. In the absence of measurements of rotations further down in the u.v. region (which are at present limited by the high absorption by the coenzyme in this region) we cannot say what the precise nature of this change is.

We are also aware that possible contributions to b_0 from excitations other than those of the helical peptide groups may arise when the inhibitor or coenzyme is bound to the protein. We consider that this is, however, unlikely on the following grounds: (1) There is no significant contribution to the rotation by the 340mμ electronic transition of the bound coenzyme, as shown by the lack of

Cotton effect in this region. One might expect this transition to be optically active if other transitions further down in the u.v. region were active. (2) It is shown below that, according to the fluorescence data, 10mol. of NADH can be bound/mol. of enzyme without affecting the value of b_0. (3) Even when NADH exhibits a large Cotton effect in the visible region on being bound (as with liver alcohol dehydrogenase), this does not lead to a change in b_0 (Rosenberg, Theorell & Yonetani, 1964). (4) Further, even if the rotatory strength of the coenzyme transitions were not low they are significantly outnumbered by the optically active transitions of the peptide chain itself, just as the aromatic side-chain transitions are insignificant for most proteins (Schellman & Schellman, 1956). (5) Beychok & Blout (1961) have shown that with myoglobin the haem chromophore makes no significant contribution to the b_0 value of the apoprotein in spite of the fact that it has a strong anomalous dispersion (Beychok & Blout, 1961). (6) The linearity of the Moffitt plots in the presence of the coenzyme and GTP with the same λ_0 value, namely 212, as used for the apoenzyme is further support for the insignificance of the contributions from the coenzyme transitions.

The effect of 1mM-GTP in the presence of concentrations of NADH (e.g. 21μM) that are alone too low to cause any change in the optical-rotatory properties of the enzyme is similar to the effect of NADH since it produces a similar change in b_0. However, under these conditions a_0 also changes (it decreases from 150 to about 50). This latter change is paralleled by a change in $S_{20,w}$, which indicates a dissociation into sub-units. In such a reversible dissociating system the value of $S_{20,w}$ cannot be taken as the final criterion of the state of aggregation. No interpretation has been made of $S_{20,w}$ values between 21·0s for the apoenzyme and 12·0s for the dissociated form characterized by light-scattering (Yielding *et al.* 1964). At lower concentrations of GTP (e.g. 0·6mM) it is possible to choose NADH concentrations that will lead to a change in b_0 but not affect a_0, and only cause some dissociation as shown by the appearance of two peaks of different mobilities (with $S_{20,w}$ values 20 and 11·5s). GTP alone does not affect the rotatory properties of the enzyme and the combined effects of GTP and NADH cannot be reversed by EDTA.

These results suggest that the changes in a_0 are related to the dissociation of the enzyme. This is reasonable, since a_0 should depend on the degree of interaction between helices and to some extent also on interactions with the solvent. Indeed, when dissociation is brought about by a non-specific reagent such as butan-2-ol, b_0 remains constant, yet a_0 again decreases. Similar observations were made by Herskovits, Townend & Timasheff (1964), who showed that a_0 is quantitatively related to the intermolecular association of β-lactoglobulin.

The results from the fluorescence measurements show that the binding constant towards NADH increases in the presence of GTP. This results in an increased fluorescence not only at low protein concentration where only a fraction of the coenzyme is bound but also at the limiting value that the curves approximate in Fig. 4. The specific fluorescence intensities of bound NADH are therefore different under the two conditions. The increased enhancement of fluorescence in the presence of GTP is consistent with a tighter binding of the coenzyme, since the enhancement is probably due to the lower ability of bound NADH to lose its energy from the excited state through non-radiative deactivation by the solvent molecules. This is in accord with Frieden's (1963) observations for NADPH, and the numerical values of the binding constants are of similar magnitude for the two systems. At the same time we find, unlike Frieden (1963), that the number of binding sites increases (cf. Tomkins *et al.* 1962), possibly as a result of dissociation. Sund (1961) found that dissociation brought about by dilution of the enzyme also results in an increase of the number of binding sites but that this non-specific dissociation leads to a diminution in the binding constant of NADH.

Our conclusions based on fluorescence measurements receive further support from the study of fluorescence polarization. The degree of polarization is a measure of the rotational motion of the bound chromophore during the life-time of the excited state. This motion is a composite of two rotations: the overall rotation of the macromolecule and the 'free' rotation of the chromophore on the surface of the macromolecule. The former should be larger for the complex of the coenzyme with the dissociated enzyme than for the complex with the aggregate. Dissociation thus should lead to a decrease in the fluorescence polarization. The observed increase therefore can be attributed to the smaller freedom of the coenzyme on the protein in the presence of GTP.

Tomkins and his collaborators have shown that the physical changes (e.g. dissociation) observable at high enzyme concentrations are related to the mechanism of non-competitive inhibition of enzymic activity under assay conditions (Yielding *et al.* 1964). Thus the conformational changes we observed are probably responsible for the inhibitions. This enzyme thus may serve as a suitable model for studying the physical basis of control of enzymic activity. It is therefore noteworthy that the conformational changes induced by NADH appear fairly sharply over a narrow range as the coenzyme concentration is increased. For example, with

26 μM-NADH there is no effect on the b_0 value of the enzyme (enzyme concentration 2 mg./ml.), yet with 95 μM-coenzyme there is a change that remains the same at higher coenzyme concentration (e.g. 133 μM). From the fluorescence-polarization data we can calculate the amount of coenzyme bound under the above conditions. Thus, when the total coenzyme concentration is 26 μM, 22 μmoles of NADH are bound/2 μmoles of glutamate dehydrogenase, whereas with 95 μM-NADH, 32 μmoles of NADH are bound at the same enzyme concentration. Therefore approx. 4–5 mol. of NADH/mol. of protein of mol.wt. 10^6 is required to induce the conformational change. The measurements do not enable us to make a more precise estimate of the NADH allosteric sites, but it appears that approx. 1 site/mol. of mol.wt. 250000 (which is the mol.wt. of the sub-unit after reversible dissociation) may be involved in the regulation of the enzymic activity.

It is tempting to speculate that whereas the regulation by NADH is of the on–off type (giving rise to the well-known sigmoid-shaped curve for allosteric inhibition) the role of GTP is to shift the threshold value at which NADH is capable of inducing the conformational change. Our results with GTP are consistent with this hypothesis.

P. M. B. thanks Merton College for a Harmsworth Senior Scholarship and Christ Church for a Research Lectureship.

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Anthraquinone Pigments from *Phoma foveata* Foister

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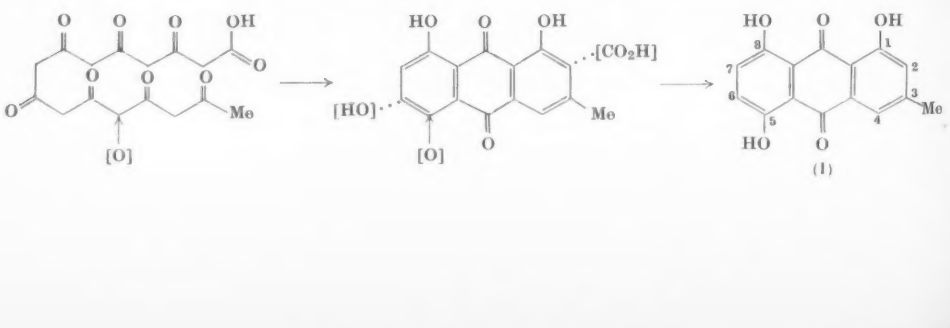
(*Received* 24 *May* 1965)

1. Suitable conditions have been determined for growing the fungus *Phoma foveata* in culture for optimum production of pigments. 2. The pigments have been extracted and fractionated according to their solubility in aqueous bicarbonate, carbonate and alkali. 3. The carbonate- and alkali-soluble pigments have been further separated by chromatography and shown to consist of anthraquinone derivatives. 4. The alkali-soluble fraction, which comprised the bulk of the pigment, consists of pachybasin (1-hydroxy-3-methylanthraquinone; II) and chrysophanol (1,8-dihydroxy-3-methylanthraquinone; III) by comparison with authentic samples. 5. The carbonate-soluble fraction contains emodin (1,6,8-trihydroxy-3-methylanthraquinone; V) and a hitherto unrecorded pigment for which the name phomarin and the structure 1,6-dihydroxy-3-methylanthraquinone (IV) are put forward. 6. The identification of emodin and the structural determination of phomarin are based largely on their ultraviolet, visible, infrared, nuclear-magnetic-resonance and mass spectra.

The imperfect fungi *Phoma solanicola* Prill. and Delacr., *Ph. tuberosa* Mel., Ros. and Sch. and *Ph. foveata* cause gangrene in potatoes, and Malcomson (1958) considers them to be indistinguishable in pathological action. The organism associated with potato gangrene in Victoria is described by Chambers (1958) as *Ph. foveata*, and this pathogen also arises periodically in Tasmania during the storage of potatoes after harvesting. During a study of this problem by P. J. Sampson (personal communication), it was observed that aging cultures of the organism formed yellow pigments that frequently crystallized as needles in the medium. We have examined these substances, and found that they belong to the class of anthraquinone pigments.

Some 40 anthraquinone derivatives of fungal or lichen origin have been described (Thomson, 1957; Joshi, 1962; Shibata, 1962). All of them have at least one α-hydroxy group, and with one exception they all contain other oxy substituents, the 1,8-dihydroxy and the 1,5,8- or 1,6,8-trihydroxy derivatives being especially common. Most of them also have a carbon-containing function, usually methyl, in the 3-position. These substitution patterns are intelligible in terms of Birch & Donovan's (1953) hypothesis of the biosynthesis of anthraquinones from a chain of acetate units, followed by cyclization and secondary reactions; the latter include decarboxylation, introduction of other oxy functions, particularly in α-positions, or loss of the 6- or 8-hydroxy groups initially present. The overall correctness of this hypothesis for helminthosporin (I) (Birch, Ryan & Smith, 1958) and for certain other anthraquinones (Gatenbeck, 1960; Shibata & Ikekawa, 1963) has been demonstrated by tracer experiments with [^{14}C]acetate. Shibata & Ikekawa (1963) have also shown that, though acetate forms the methyl end of the polyacetate chain, the remainder is formed preferentially from malonate. From analogy with fatty acid biosyn-



(I)

thesis, it may be inferred that acetate must first be carboxylated to malonate before incorporation into the remainder of the chain.

MATERIALS AND METHODS

Melting points are corrected. Evaporations were carried out *in vacuo*, and sublimations at 105° and 10^{-6}mm. Chromatographic solvents were purified by distillation; column chromatography was carried out on $MgCO_3$ ('heavy'; May and Baker Ltd., Dagenham, Essex)–Hyflo Super-Cel (Hopkin and Williams Ltd., Chadwell Heath, Essex) (1:1, w/w); for thin-layer chromatography a slurry of water–$MgCO_3$ (1:1, w/w) (without binder) was spread as thinly as possible for maximum resolution on 8in.^{2} plates, which were air-dried for 24hr. and stored over silica gel. They were developed with benzene–chloroform mixtures, and at very low concentrations the positions of the pigments were observed under u.v. light. The u.v. and visible spectra were determined in ethanol on a Perkin–Elmer model 4000A Spectracord Spectrophotometer; i.r. spectra were determined in Nujol mulls, or in hexachlorobutadiene in the regions of Nujol absorption, on a Perkin–Elmer model 221 Spektrophotometer; n.m.r. (nuclear-magnetic-resonance) spectra were determined on a Varian model A60 Spectrometer, with tetramethylsilane as internal reference; mass spectra were determined on a Metropolitan–Vickers model M.S.9 Mass Spectrometer with direct entry system. Microanalyses were carried out by the C.S.I.R.O. Microanalytical Service, Melbourne, Victoria, Australia.

The fungus was grown initially on potato–dextrose agar in 9cm.-diam. glass Petri dishes, but a modified Sabouraud medium (Robinow, 1963) was later adopted. After 9 days' growth at room temperature without temperature control, the contents of the dishes were dried *in vacuo* at 60°, ground in a Wiley no. 1 mill and extracted in a Soxhlet apparatus with acetone until no further coloured materials were removed. An extract from 400 plates produced 10g. of sticky red crystalline material on evaporation. This was dissolved in benzene and the solution extracted in a separating funnel with aq. 5% (w/v) NaOH until no further red salts were removed. The alkaline solution was acidified with HCl and the regenerated yellow pigments were extracted with chloroform. The solvent was removed and replaced with benzene, and the solution extracted successively with aq. 5% (w/v) $NaHCO_3$ and aq. 5% (w/v) Na_2CO_3. After these extractions, the bulk of the alkali-soluble pigment remained in the benzene solution, which on evaporation yielded fraction R_1. The aqueous solutions were separately acidified and extracted with chloroform as before; the chloroform solution thus obtained from the carbonate extract yielded fraction R_2 on evaporation, and that from the bicarbonate extract fraction R_3.

Fraction R_1 was chromatographed on a column in light petroleum (b.p. 40–60°). Two coloured bands appeared, and on elution with acetone the lower straw-yellow band (R_1A) was completely removed. The pigment in the remaining pink band (R_1B) was recovered by dissolving the $MgCO_3$ from the column in dilute HCl, then extracting the mixture with chloroform. The acetone and chloroform extracts were evaporated separately, and the homogeneity of the fractions R_1A and R_1B thus obtained was checked by thin-layer chromatography; the fractions were finally purified by sublimation.

Fraction R_2, dissolved in light petroleum–acetone (3:1, v/v), was chromatographed similarly to R_1. Eight bands appeared, of which R_2A, the most easily eluted, proved to be identical with R_1A. This was followed by R_2B (pale pink), R_2C (yellow), R_2D (pale pink) and R_2E (pink). The column was cut into suitable sections and each pigment recovered as before. Fractions R_2B, R_2C and R_2E only were present in sufficient quantity (1–5mg.) for further investigation; they were purified as described above.

Fraction R_3 has not been examined in detail, but column chromatography revealed at least nine coloured bands, one of which was eluted by benzene, and proved to be soluble in aq. 5% (w/v) NaOH but insoluble in aq. 5% (w/v) $NaHCO_3$ or aq. 5% (w/v) Na_2CO_3. The amount was insufficient to verify its identity with pigment R_1A, with whose behaviour it corresponds.

RESULTS

Characterization of pigments. Fractions R_1A, R_1B, R_2B, R_2C and R_2E all gave negative zirconium nitrate reactions (Feigl, 1960), indicating the absence of hydroxyl groups *ortho* to one another. Fraction R_1A was crystallized from methanol and finally obtained as a yellow sublimate, m.p. 176° unchanged by admixture with an authentic sample of pachybasin (II), which was synthesized by the method of Waldmann & Sellner (1938). Light-absorption maxima were ($\log \epsilon_{max.}$ in parentheses): 224 (3·74), 252 (4·01), 281 (3·65) and 403mμ (3·02); the i.r.-absorption maxima were: 3083, 2967, 2933, 2862, 1677, 1642, 1593, 1578, 1403, 1380, 1338, 1311, 1290, 1235, 1148, 1068, 1039, 1002, 929, 872, 805, 793, 769 and 712cm.$^{-1}$. The n.m.r. spectrum in $CDCl_3$ showed δ: 2·45 (Me), 7·17 (H-2; broad, 4 cyc./sec.), 7·55 (H-4; broad, 4 cyc./sec.), 8·20 (H-5 and H-8), 7·74 (H-6 and H-7) and 12·67p.p.m. (OH) (Found: C, 75·9; H, 4·3; O, 20·8. Calc. for $C_{15}H_{10}O_3$: C, 75·6; H, 4·2; O, 20·2%).

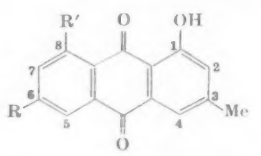
Fraction R_1B formed orange–yellow crystals on sublimation, m.p. 186° alone and 189° on admixture with an authentic sample (m.p. 193°) of chrysophanol (III) (supplied by Fluka A.-G., Buchs, Switzerland, and purified by chromatography as above). The i.r. spectra of R_1B and chrysophanol were identical (Bloom, Briggs & Cleverley, 1959), as also were the u.v. and visible spectra (Birkinshaw, 1955). The n.m.r. spectrum in $CDCl_3$ showed δ: 2·45 (Me), 7·12 (H-2; broad, 4cyc./sec.), 7·40 (H-4; broad, 3cyc./sec.), 7·80 (H-5, H-6 and H-7), 12·00 (OH) and 12·14p.p.m. (OH) (Found: C, 71·0; H, 4·1; O, 25·3. Calc. for $C_{15}H_{10}O_4$: C, 70·9; H, 3·9; O, 25·2%).

Fractions R_2B, R_2C and R_2E were not available in sufficient quantity for rigorous purification. The

first two had similar u.v. and visible spectra, the same n.m.r. spectra, and the same parent peaks and similar fragmentation patterns in their mass spectra. It is considered that they consist of the same substance, *phomarin*, contaminated with different impurities. From the mass spectra, R_2B appeared to contain a higher-molecular-weight waxy substance, and R_2C apparently contains small amounts of R_2E. After further purification by chromatography and sublimation, fraction R_2B was used to determine the light-absorption spectra for phomarin, and R_2C for the remaining data.

Phomarin forms a yellow crystalline sublimate that decomposes gradually above 200° without melting. It dissolves in aq. 5% (w/v) sodium carbonate almost without colour change. The light-absorption maxima were: 219, 268, 293 (shoulder) and 410 mμ; after being refluxed with 1 ml. of acetic acid and 1 ml. of 48% (w/v) hydrobromic acid for 7½ hr., the spectrum of phomarin (0·5 mg.) was unchanged. The i.r.-absorption maxima were: 3414, 3310, 2925, 2856, 1677, 1640, 1603, 1579, 1488, 1389, 1305, 1268, 1191, 1153, 1020, 945, 894, 854, 817, 795 and 795 cm.$^{-1}$. The n.m.r. spectrum in acetone showed δ: 7·10 (broad, 3 cyc./sec.), 7·56 (broad, 3 cyc./sec.), 7·28 (quartet, J_1 8·5, J_2 2·5 cyc./sec.), 7·65 (doublet, J 2·5 cyc./sec.) and 8·14 p.p.m. (doublet J 8·5 cyc./sec.) (Found: mol.wt. from mass spectrum: 254·0561. $C_{15}H_{10}O_4$ requires 254·0579).

Fraction R_2E was obtained as an orange sublimate that was rechromatographed on a column as before. Two small leading bands (red and violet) were discarded, and the main pink band was cut into three equal sections that were extracted as described above. The three fractions had nearly identical i.r. spectra, which were substantially the same as the recorded spectrum of emodin (Bloom, *et al.* 1959). The u.v. and visible spectra of R_2E were identical with those of emodin and citreorosein (Birkinshaw, 1955). The m.p. of R_2E was 240° and a mixed m.p. with an authentic sample (m.p. 255°) of emodin (supplied by Fluka) was 251°. The n.m.r. spectrum in acetone showed δ: 7·02 (broad, 3 cyc./sec.), 7·49 (broad, 3 cyc./sec.), 7·19 (doublet, J 2·5 cyc./sec.), 6·57 (doublet, J 3 cyc./sec.) and 7·77 p.p.m.; relative intensities were 20, 27, 31, 25 and 15 (Found: mol.wt. from mass spectrum: 270·0519. Calc. for $C_{15}H_{10}O_5$: 270·0528). In the mass spectrum, in addition to the main parent peak, there was a small peak at mass 254 and an even smaller one at mass 286.



(II) R = R′ = H
(III) R = H, R′ = OH
(IV) R = OH, R′ = H
(V) R = R′ = OH
(VI) R = OMe, R′ = OH
(VII) R = O·CH_2·CH:CMe_2, R′ = OH

DISCUSSION

Considerable advantages were found in the use of modified Sabouraud medium (Robinow, 1963) over potato–dextrose agar medium in growing the fungus: the time required to achieve maximum pigment production was thereby reduced from 21 to 9 days, and at the same time considerably more pigment was produced. However, the capacity to produce pigments was rapidly lost on continued subculturing on artificial media, and stock cultures were maintained on potatoes (var. Pink Eye) at 4°. Pigment production in liquid potato–dextrose medium was poor, and the extraction procedure tedious owing to the formation of emulsions.

The two major pigments, pachybasin (R_1A; II) and chrysophanol (R_1B; III) were obtained substantially pure and readily identified, but the separation procedures were inadequate for rigorous purification of the minor components. It was clear that the presence of mixtures affected the solubility in alkali of some of the components: thus pachybasin, which when pure is insoluble in carbonate and bicarbonate solutions, was present in the former extract and probably in the latter as well. Moreover, it was evident that discrete coloured bands appearing in a chromatograph column did not necessarily correspond to separate compounds; the bands could be due to mixtures of the same components with different amounts of impurities, as observed in other anthraquinone studies (R. G. Cooke, personal communication).

From its mass spectrum, fraction R_2E consists very largely of a compound $C_{15}H_{10}O_5$, with smaller amounts of $C_{15}H_{10}O_4$ and even less of $C_{15}H_{10}O_6$. The spectral data indicate that R_2E consists mainly of emodin; its melting point is low for emodin, but its identity is confirmed by the following considerations. The visible spectrum of R_2E has an absorption maximum at 435 mμ, which indicates the presence of two α-hydroxyl groups (Briggs, Nicholls & Paterson, 1952; Birkinshaw, 1955). These must be in the 1,8-positions, since a 1,4- or 1,5-arrangement would produce 'conjugate chelation' (Bellamy, 1958; Bloom *et al.* 1959) in both carbonyl groups; the carbonyl absorptions (1677 and 1625 cm.$^{-1}$) in the infrared spectrum of R_2E show in fact that only one is thus chelated. The aromatic

portion of the n.m.r. spectrum of fraction R_2E indicates that four aromatic protons are present in the nucleus, two in each ring; further, they are in *meta* positions in each case, since they exhibit long-range coupling with a small coupling constant (Jackman, 1959). The substitution pattern is thus at positions 1, 3, 6 and 8, as for physcion (VI) and madagascin (VII), whose published n.m.r. spectra (Ritchie & Taylor, 1964*a*,*b*) correspond to that of R_2E in the aromatic region. From the molecular formula $C_{15}H_{10}O_5$ of the major component of R_2E, the two other substituents must be a methyl group and a hydroxyl group, and thus R_2E consists principally of emodin (V). The main impurity in R_2E is evidently chrysophanol (III) from its molecular formula $C_{15}H_{10}O_4$, and from the occurrence of a small peak at δ 7·77 p.p.m. in the n.m.r. spectrum of fraction R_2E, which corresponds to the absorption of the three protons attached to positions 5, 6 and 7 of chrysophanol; the remaining aromatic protons at positions 2 and 4 in the latter give absorptions that overlap with some of the peaks for emodin.

The n.m.r. spectrum of phomarin indicates the presence of five aromatic protons; there are thus three substituents, and, since the molecular formula is $C_{15}H_{10}O_4$, these must be two hydroxyl groups and a methyl group. This conclusion is supported by a comparison of the fragmentation patterns obtained from chrysophanol and phomarin by mass spectroscopy. The main peaks were in corresponding positions although they differed in intensity. A peak at mass 239 $(p-15)^+$ is attributed to loss of a methyl group from the parent ion, one of mass 237 $(p-17)^+$ to loss of a hydroxyl group and one of mass 169 to the fragment $(p-C_3O_3H)^+$, which was recorded in the mass spectra of all dihydroxyanthraquinones examined by Beynon & Williams (1960). From the carbonyl absorptions (1677 and 1640 cm.$^{-1}$) in the i.r. spectrum of phomarin, one of the hydroxyl groups is in an α-position (Bloom *et al.* 1959), and from the very strong aromatic bands in the 1560–1620 cm.$^{-1}$ region, more intense than the carbonyl bands, the other hydroxyl group is in a β-position (Tanaka, 1958). These assignments for the two hydroxyl groups are confirmed by the light-absorption peak at 410 mμ, which indicates that one hydroxyl group only is in an α-position (Briggs *et al.* 1952; Birkinshaw, 1955). The hydroxyl groups cannot be in the same ring, otherwise the protons attached to it would produce doublets in the n.m.r. spectrum, and the aromatic region would show not more than one singlet absorption. Two broad singlets in fact appear at δ 7·10 and 7·56 p.p.m. (each 3 cyc./sec.); they are present in approximately the same positions in all the spectra of the anthraquinones from *Ph. foveata*, and also in the recorded spectra of physcion (VI) and madagascin (VII) (Ritchie & Taylor, 1964*a*,*b*). These absorptions seem to be typical of *meta*-grouped protons H-2 and H-4 exhibiting long-range coupling through a methyl group. The spectral evidence thus places a methyl group *meta* to an α-hydroxyl group, and points to phomarin having a 1,6- or 1,7-dihydroxy-3-methylanthraquinone structure. This is further confirmed by the remaining aromatic portion of the n.m.r. spectrum. The quartet at δ 7·28 p.p.m. (J_1 8·5, J_2 2·5 cyc./sec.) is assigned to a β-proton split by both α-protons attached to the same ring, one *ortho* and one *meta* to it; the β-proton in its turn splits each of the α-ones, one *ortho* at δ 8·14 p.p.m. (J 8·5 cyc./sec.) and one *meta* at δ 7·65 p.p.m. (J 2·5 cyc./sec.). The n.m.r. evidence does not differentiate between the above-mentioned alternative structures for phomarin, but a distinction can be drawn from their recorded i.r. spectral data, which are limited to the 1560–1620 cm.$^{-1}$ region: a Nujol mull of the 1,7-dihydroxy compound showed one very strong absorption band at approx. 1580 cm.$^{-1}$, whereas for the 1,6-dihydroxy compound the aromatic absorption was split into two bands at approx. 1600 and 1580 cm.$^{-1}$ (Tanaka, 1958). Phomarin shows two intense bands at 1603 and 1579 cm.$^{-1}$ in this region, and it is therefore assigned the structure 1,6-dihydroxy-3-methylanthraquinone (IV). This structure is also preferred on biogenetic grounds, and on this basis both phomarin and chrysophanol have deoxyemodin structures.

Pachybasin (II) and chrysophanol (III) have been isolated previously from the fungus *Pachybasium candidum* (Sacc.) Peyronel by Shibata & Takido (1955), and chrysophanol in addition from *Penicillium islandicum* Sopp. by Howard & Raistrick (1950). Emodin (V) has been isolated from the latter mould (Shibata, 1962), and also from *Cortinarius sanguineus* (Wulf) Fries (Kögl & Postowsky, 1925) and from a mutant strain of *Cladosporium fulvum* Cooke (Agosti, Birkinshaw & Chaplen, 1962). In addition, emodin and chrysophanol have been found in many higher plants (Karrer, 1958).

We are grateful to Mr P. J. Sampson, Department of Agriculture, Hobart, for suggesting this problem and for providing cultures and mycelium, and to Dr R. G. Cooke, Chemistry Department, University of Melbourne, for the gift of a sample of chrysophanol. We also thank Dr Q. N. Porter of the same Department, and Dr A. J. C. Nicholson and his colleagues of the Division of Chemical Physics, C.S.I.R.O., Melbourne, who made the mass-spectra determinations; also Dr T. J. Batterham, Division of Organic Chemistry, C.S.I.R.O., Melbourne, who determined the n.m.r. spectra and who furnished much helpful advice. Our thanks are also due to the Commonwealth Government for a scholarship to C. R.

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Effect of Lipids and Organic Solvents on the Enzymic Formation of Zinc Protoporphyrin and Haem

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(*Received* 15 *April* 1965)

1. Differences observed in earlier work between the enzymic chelation with protoporphyrin of Zn^{2+} and Fe^{2+} ions respectively have now been explained as being caused by the presence of peroxides in the ether used in the enzyme assay. The inhibitory effect of peroxides is established by the reducing agent, which is present in the assay for chelation of iron but not in that for zinc. There are now no reasons for the belief that two different enzymes catalyse formation of complexes with zinc and iron respectively. 2. Removal of lipid from both chromatophores and mitochondria markedly reduced chelatase activity. Activity could be partially restored by the addition of lipid fractions. Phosphatidic acid, but not phosphatidylcholine or phosphatidylethanolamine, actively stimulated the formation of zinc protoporphyrin and haem by chromatophores and mitochondrial preparations. 3. Lipid-containing extracts of chromatophores, and fractions thereof obtained by silicic acid chromatography, partially restored chelatase activity of Tween extracts of mitochondria. Thus, although both enzymes are considered to be lipoproteins, the identity of the lipids concerned is still uncertain. 4. A great number of organic solvents such as esters, ethers, ketones and, to a lesser extent, alcohols, stimulate enzymic chelation of both metals with protoporphyrin. A number of explanations for these findings are considered and it is suggested that organic solvents interact in some way with the enzyme lipoprotein, changing either its conformation or allowing closer contact between the enzyme and its substrates.

Neuberger & Tait (1964) reported the presence of an enzyme which catalysed the formation of zinc protoporphyrin in chromatophores of *Rhodopseudomonas spheroides* and in mitochondria from a number of mammalian tissues. They discussed whether this enzyme might be identical with the ferrochelatase present in the same sources. Some of the properties of these chelatases were studied and, in particular, a marked stimulation was noted when the reactions were brought about in water–ether emulsion.

In this paper we report further properties of both non-enzymic and enzymic systems which form zinc protoporphyrin and haem. Other organic solvents, in addition to ether, have been found to stimulate the enzymic reactions with both chromatophores and mitochondria. It was found that phosphatidic acid stimulates the enzymic reaction. The chelatase in mitochondria from guinea-pig and pig liver has been brought into solution and, in this form, its activity is stimulated by a lipid extract obtained from chromatophores of *R. spheroides*. The evidence reported here also leads us to believe that both zinc–protoporphyrin-chelatase and ferrochelatase activities are catalysed by the same enzyme. Generally assays have been done with zinc, and not with iron, because assays with the former are easier to perform and are more sensitive, but all the more important results have been obtained with both metals.

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MATERIALS AND METHODS

Phosphatidylethanolamine and phosphatidylcholine were prepared from egg yolk by the method of Rhodes & Dawson (1960). Phosphatidic acid was prepared from phosphatidylcholine by hydrolysis with a partially purified phospholipase D (EC 3.1.4.4) from cabbage (Davidson & Long, 1958), and was purified by chromatography on silicic acid followed by precipitation as the sodium salt (Kates, 1955). Trypsin, chymotrypsin and bovine plasma albumin were obtained from Armour and Co. Ltd., Eastbourne, Sussex. Organic solvents used in the enzyme assays were dried over Na_2SO_4, since it was found that a number of them, if used without drying, caused zinc protoporphyrin formation in the absence of added $ZnSO_4$. This did not occur with solvents

which had been dried before being added to the enzyme assays. Peroxide-free ether was prepared by the method of Vogel (1954).

Enzyme sources. Chromatophores were prepared according to Gibson, Neuberger & Tait (1963) from *R. spheroides*. The organisms used were grown semi-anaerobically in the light in the malate medium of Lascelles (1959).

Mitochondria were prepared from guinea-pig or pig liver essentially by the method of Hogeboom (1955). After removal of nuclei from the homogenate by centrifuging at low speed, the supernatant was centrifuged at 15000*g* for 10min. The supernatant and the loosely packed pink pellet were removed and the tightly packed brown pellet was washed, by suspending in 0·25M-sucrose and recentrifuging, to give 'heavy' mitochondria. The 15000*g* supernatant, including the loosely packed pink pellet, was centrifuged at the same speed for 20min. to give a pink pellet which was washed by resuspending and recentrifuging to give 'light' mitochondria. The 'light' fraction is probably a mixture of lysosomes (cf. Appelmans, Wattiaux & de Duve, 1955) and mitochondria which have been damaged during the isolation (cf. Hatefi & Lester, 1958).

Estimations and chromatographic methods. Estimations of protein and porphyrins, and of zinc–protoporphyrin-chelatase and ferrochelatase activities, were performed as described by Neuberger & Tait (1964). Lipids were extracted from aqueous suspensions of fractions with chloroform–methanol mixtures (Bligh & Dyer, 1959). Samples of lipids were digested and their phosphorus content was estimated according to Bartlett (1959).

Column chromatography of lipids was performed on silicic acid (100mesh; Mallinckrodt Chemical Works, U.S.A.) activated by heating at 100° overnight before use.

Thin-layer chromatography of lipids was performed on 20cm.×20cm. glass plates coated with Keiselgel G (E. Merck A.-G., Darmstadt, Germany) and developed with chloroform–methanol–water (65:25:4, by vol.; Wagner, Hörhammer & Wolff, 1961). Lipids were located by exposure of the dried plates to iodine vapour (Sims & Larose, 1962), or by spraying with Rhodamine 6 G (Marinetti, Erbland & Kochen, 1957), or by spraying with 10N-H_2SO_4 followed by heating at 160° (Wagner *et al.* 1961). Constituents containing free amino groups were detected with ninhydrin. The identity of the lipids in mixtures was established by comparison of their R_F values with those of pure lipids prepared from egg yolk (see above) and with R_F values given in the literature (cf. Lepage, 1964).

RESULTS

Non-enzymic formation of zinc protoporphyrin. Neuberger & Tait (1964) have shown that under the conditions used in the enzyme assays (0·2M-tris buffer, pH8·4, 0·08mM-protoporphyrin, 0·06mM-zinc chloride, incubated at 37°) non-enzymic formation of zinc protoporphyrin was slow as compared with enzymic formation, and slow compared with the non-enzymic formation of zinc complexes of deuteroporphyrin, mesoporphyrin, haematoporphyrin and coproporphyrin. It was also shown that, unlike the enzymic reaction, the non-enzymic reaction was not stimulated by ether. If the pH was raised above 10, the rate of formation of zinc protoporphyrin increased markedly (Fig. 1). This was found in tris or triethanolamine buffers, but not in carbonate–bicarbonate or glycine–sodium hydroxide buffers of similar pH values. In tris buffer with the addition of sodium lauryl sulphate (Fig. 1) the reaction proceeded more rapidly, and even at lower pH values. The rate at a given pH value was dependent on the concentration of sodium lauryl sulphate up to 0·5% (w/v); higher concentrations did not stimulate the reaction further. Several anionic, cationic and non-ionic detergents, such as sodium deoxycholate, cetyltrimethylammonium bromide, acetylpyridinium bromide, Tween 20 and Tween 80 had no effect on zinc protoporphyrin formation, when used at a concentration of 0·5% (w/v) in place of sodium lauryl sulphate. Different pH–activity curves were obtained in the presence of sodium lauryl sulphate when triethanolamine was used

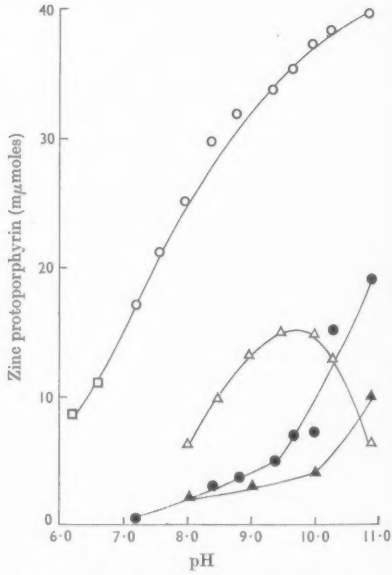

Fig. 1. Effect of pH in different buffers on the non-enzymic formation of zinc protoporphyrin in the presence and absence of sodium lauryl sulphate. Buffer (tris, ○; triethanolamine, △; maleate, □) of pH stated (100μmoles), protoporphyrin (45mμmoles) and $ZnSO_4$ (40mμmoles) were incubated in a total volume 0·5ml. at 37°. The incubation was for 45min. when sodium lauryl sulphate (2·5mg.) was present (○, △, □) and for 90min. when it was not used (●, ▲). The amounts of zinc protoporphyrin formed were measured as described by Neuberger & Tait (1964).

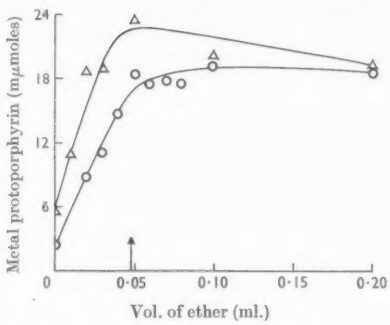

Fig. 2. Effect of diethyl ether on the formation of zinc protoporphyrin and haem catalysed by chromatophores. Tris, pH 8·4 (100 μmoles), protoporphyrin (42 mμmoles), chromatophores (1·1 mg. of protein) and either $ZnSO_4$ (40 mμmoles) or $FeSO_4$ (100 mμmoles) plus ascorbic acid (10 μmoles) were mixed in a volume 0·5 ml. The volume of ether stated was added, the tubes were tightly stoppered, shaken and incubated at 32° for 1 hr. Zinc protoporphyrin (○) and haem (△) were measured as described by Neuberger & Tait (1964). The arrow denotes the formation of a two-phase system.

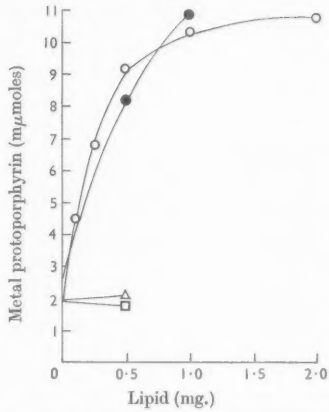

Fig. 3. Effect of lipids on zinc protoporphyrin and haem formation catalysed by chromatophores. Phosphatidic acid (○ and ●) or phosphatidylethanolamine (△) or phosphatidylcholine (□) were added in ether solution to the tubes, and the ether was evaporated by a stream of nitrogen. To the tubes were added tris, pH 8·4 (100 μmoles), protoporphyrin (40 mμmoles), either $ZnSO_4$ (40 mμmoles) or $FeSO_4$ (100 mμmoles) plus ascorbic acid (10 μmoles), chromatophores (0·9 mg. of protein) and water to 0·5 ml. The lipids were emulsified by vigorous manual shaking and the samples incubated at 37° for 1 hr. Zinc protoporphyrin (○, □, △) and haem (●) were measured as described by Neuberger & Tait (1964).

Table 1. *Effect of organic solvents on zinc–protoporphyrin-chelatase activity in chromatophores*

Tris, pH 8·4 (100 μmoles), protoporphyrin (42 mμmoles), $ZnSO_4$ (40 mμmoles), chromatophores (1·5 mg. of protein) and water were made to 0·5 ml. Organic solvent (0·02 ml., except where otherwise stated) was added and after thorough mixing incubation was carried out at 37° for 1 hr. The porphyrins were isolated and the amount of zinc protoporphyrin formed was estimated as described by Neuberger & Tait (1964).

Solvent	Zinc protoporphyrin (mμmoles/assay)	Solvent	Zinc protoporphyrin (mμmoles/assay)
None	3·2	Acetone (0·1 ml.)	12·0
Ether (0·2 ml.)	14·6	Ethyl methyl ketone (0·04 ml.)	13·0
Methyl acetate (0·07 ml.)	16·5	Ethylene glycol monoethyl ether	5·2
Ethyl acetate	18·1	Dioxan	8·7
Ethyl aceto-acetate	16·5	Ethanol	5·9
Ethyl chloro-acetate	14·1	Propanol	7·3
Ethyl formate	12·0	Butanol	6·1
Diethyl malonate	11·2	Hexane	4·1

instead of tris (Fig. 1). None of the organic solvents used in the enzymic work (see below) had any stimulatory effect in the absence of enzyme, as shown in experiments carried out in various buffers at high pH values and with the various detergents mentioned above.

Enzymic formation of zinc protoporphyrin and haem catalysed by chromatophores. Neuberger & Tait (1964) showed that the rate of formation of zinc protoporphyrin and haem, catalysed by chromatophores, was increased by a factor of about six when the assays were carried out in a water–ether emulsion. Fig. 2 shows that the stimulation was linearly proportional to the concentration of ether up to saturation; addition of more ether had no further effect. A similar increase in the rate of the reaction occurred when ether was replaced by ketones or esters, whereas alcohols and hydrocarbons had little effect (Table 1). The effect of a number of ketones and esters was studied in more detail and it was found that maximum stimulation was caused by addition of 0·1 ml. of acetone, 0·04 ml. of ethyl methyl ketone or 0·03 ml. of ethyl acetate to the 0·5 ml. of aqueous reaction mixture; at higher concentrations the rate of the reaction was reduced.

At optimum concentrations of each organic solvent the rate of the reaction was increased to about the same extent.

In the presence of organic solvents, as in their absence (Neuberger & Tait, 1964), the rate of the reaction was linear with increasing amounts of chromatophore protein per assay up to 1·2mg.

None of the above compounds had any effect on the rate of the non-enzymic formation of zinc protoporphyrin. The same detergents examined for an effect on the non-enzymic formation of zinc protoporphyrin were tested on the enzymic reaction. Tween 20 and 80 stimulated by a factor of two but the others were without effect. Any action of sodium lauryl sulphate on the enzymic reaction was obscured by its large effect on the non-enzymic reaction.

The effect of a number of phospholipids, prepared from egg yolk, was tested. Phosphatidic acid stimulated the production of zinc protoporphyrin and haem (Fig. 3), but phosphatidylcholine and phosphatidylethanolamine were inactive. The amount of phosphatidic acid required to give maximum stimulation increased with the amount of chromatophores used. When the reaction was performed in the presence of phosphatidic acid or phosphatidic acid plus ether, addition of excess of zinc sulphate did not inhibit the reaction as it did in the presence of ether only. Thus, when chromatophores (1·4mg. of protein) were assayed with 50mμmoles and 150mμmoles of zinc sulphate, the amounts of zinc protoporphyrin formed in 90min. were 14·6 and 6·3mμmoles respectively in the presence of ether (0·2ml.), 10·4 and 10·6mμmoles respectively in the presence of phosphatidic acid (1·5mg.) and 23·4 and 21·5mμmoles respectively in the presence of ether plus phosphatidic acid. From these results it can be seen that phosphatidic acid stimulates activity even in the presence of ether.

Attempts to extract the enzyme from chromatophores with a number of detergents were unsuccessful. Thus chromatophores were frozen and thawed in the presence of Tween 20 (10mg./ml.) and then centrifuged at 105000*g* for 1hr. Only low enzymic activity was detected in the supernatant, or the pellet (chromatophore-Tween pellet), or when the two fractions were combined.

Table 2. *Effect of extracting chromatophores with acetone on the zinc–protoporphyrin-chelatase activity*

Acetone-dried powder was prepared from chromatophores by the method of Morton (1955). It was suspended in 0·05M-tris, pH7·4, before use. Chromatophores and acetone-dried powder were extracted with 90% (v/v) acetone at 0° as described by Lester & Fleischer (1961). The pellet was dried and suspended in 0·05M-tris, pH7·4. The 90% (v/v) acetone extract was evaporated to dryness under N_2 and the residue dissolved in a small volume of dry ether. Chromatophore-Tween pellet was prepared as described in the text. Assay: tris, pH8·4 (100μmoles), protoporphyrin (47·5mμmoles), $ZnSO_4$ (50mμmolcs), preparations as described above, and water were made to 0·5ml. Ether (0·2ml.) or acetone (0·1ml.) was added and, after mixing, incubation was carried out for 60min. at 37°. The amount of zinc protoporphyrin formed was estimated as described by Neuberger & Tait (1964).

Preparation	Additions	Zinc protoporphyrin (mμmoles) Ether	Acetone
Chromatophores (2·15mg. of protein)	None	31·8	—*
Acetone-dried powder (2·05mg. of protein)	None	12·8	17·9
Acetone-dried powder (2·05mg. of protein)	Chromatophore-Tween pellet (0·36mg. of protein)	22·9	16·4
Acetone-dried powder (1·56mg. of protein)	None	5·5	—
Acetone-dried powder (1·56mg. of protein)	Lipid extracted from chromatophores with $CHCl_3$–methanol (0·19μmole of lipid phosphate)	9·8	—
Powder prepared by extracting acetone-dried powder with 90% (v/v) acetone (1·71mg. of protein)	None	1·0	3·4
	Chromatophore-Tween pellet (0·55mg. of protein)	20·0	20·7
	Lipid extracted with 90% (v/v) acetone (0·55μmole of lipid phosphate)	9·0	—
None	Chromatophore-Tween pellet (0·55mg. of protein)	4·0	2·6
Powder prepared by extracting chromatophores with 90% (v/v) acetone (1·67mg. of protein)	None	6·0	13·4
	Chromatophore-Tween pellet (0·55mg. of protein)	18·7	19·2

* Not measured.

The activation of the chelatases by organic solvents, their inactivation by detergents, and the fact that chromatophores have a high content of lipid (cf. Newton & Newton, 1957) suggested that lipid played some role in these reactions. An acetone-dried powder of chromatophores had a lower zinc–protoporphyrin-chelatase activity than untreated chromatophores; further, the activity of the acetone-dried powder was increased by chromatophore-Tween pellet (prepared as described above) or by lipids extracted from chromatophores with chloroform–methanol mixture (Table 2). This acetone-dried powder was green, showing that all the bacteriochlorophyll had not been removed, and chromatography of an extract made with chloroform–methanol showed that a considerable amount of phospholipid had remained after acetone extraction. The acetone-dried powder and untreated chromatophores were extracted with 90% (v/v) acetone at 0°, a method reported by Lester & Fleischer (1961) to remove most of the phospholipid of mitochondria. Such preparations were colourless and had very low activity when assayed in the presence of ether or acetone; this activity was greatly increased by adding a chromatophore-Tween pellet or an ether solution of the lipid extracted with 90% (v/v) acetone (Table 2).

The ability of chromatophores to catalyse the formation of zinc protoporphyrin and haem was markedly reduced by preincubation with either trypsin or chymotrypsin. For example the zinc–protoporphyrin-chelatase activity of chromatophores (1·5mg. of protein) fell by about 50% on incubation at pH 8·4 and at 37° for 1hr. with trypsin (60μg.).

Effect of peroxides on chelatase activity in mitochondria. Ether and other organic solvents stimulated chelatase activity with mitochondria as they did with chromatophores. However, when mitochondria were assayed for zinc–protoporphyrin-chelatase activity in the presence of ether, reproducible results were not obtained; in addition the activity did not increase linearly with increasing amounts of mitochondria; the activity per mg. of protein was lower with small amounts of mitochondria and higher with large amounts of mitochondria.

Porra & Jones (1963*a*) and Labbe & Hubbard (1960) showed that the ferrochelatase activity appeared in the supernatant after centrifuging (36000*g* for 1hr.) a preparation of mitochondria previously frozen and thawed in Tween 20 (10mg./ml.). Such supernatants also showed zinc–protoporphyrin-chelatase activity (Neuberger & Tait, 1964). In the present work the mitochondria, after treatment with Tween 20, were centrifuged at 105000*g* for 1hr. and both activities were still present in the supernatant.

When this supernatant was assayed for zinc–protoporphyrin-chelatase activity in the presence of ether, results similar to those described above with whole mitochondria were obtained (Fig. 4). When chromatophore-Tween pellet (prepared as described above) was added, the activity was increased, particularly when small amounts of mitochondrial supernatant were used, and the enzyme–activity curve was now linear (Fig. 4). Stimulation of enzyme activity was also obtained by adding ascorbic acid to the assay. When assays were done in the presence of acetone reproducible results were always obtained and the addition of chromatophore-Tween pellet and ascorbic acid did not have marked effects on the activity. Reproducible results and linear enzyme–activity curves were usually obtained for

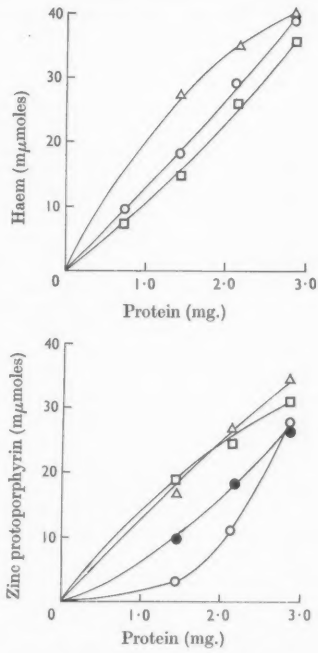

Fig. 4. Enzyme–activity curves for zinc protoporphyrin and haem formation catalysed by guinea-pig liver 'light'-mitochondrial-Tween supernatant. Tris, pH 8·4 (100μ-moles), protoporphyrin (47·5mμmoles), either $ZnSO_4$ (50mμmoles) or $FeSO_4$ (100mμmoles) plus ascorbic acid (10μmoles), 'light'-mitochondrial-Tween supernatant (as stated) and chromatophore pellet (when added, 0·36mg. of protein) were mixed in a volume 0·5ml. Ether (0·2ml.) or acetone (0·1ml.) was added and, after mixing, the samples were incubated at 37° for 1hr. Zinc protoporphyrin and haem were measured as described by Neuberger & Tait (1964). ○, Ether; △, ether + chromatophore pellet; □, acetone; ●, ether + ascorbic acid (10μmoles).

Table 3. *Activity of zinc–protoporphyrin chelatase in supernatant from Tween-treated guinea-pig-liver mitochondria when assayed in the presence of different compounds*

Supernatant from Tween-treated guinea-pig-liver mitochondria was prepared as described in the text. Assay: tris, pH 8·4 (100 μmoles), protoporphyrin (50 mμmoles), $ZnSO_4$ (50 mμmoles), supernatant from Tween-treated mitochondria (as stated) and, where added, ascorbic acid (10 μmoles) and chromatophore pellet (0·18 mg. of protein) were mixed in 0·5 ml. of water. Freshly dried ether (0·2 ml.) or peroxide-free ether (0·2 ml.) or acetone (0·1 ml.) was added and, after mixing, the tubes were incubated at 37° for 1 hr. The amount of zinc protoporphyrin formed was estimated as described by Neuberger & Tait (1964).

Additions to assay	Mitochondrial-Tween supernatant (mg. of protein/assay)	Zinc protoporphyrin (mμmoles) 0·48	0·80	0·96	1·44
Ether		2·5	7·0	15·0	31·8
Ether + chromatophore pellet		13·9	25·5	26·4	36·3
Ether + ascorbic acid		7·5	21·8	20·5	34·8
Ether + ascorbic acid + chromatophore pellet		18·8	—*	36·3	39·3
Peroxide-free ether		12·1	19·0	23·1	35·0
Peroxide-free ether + ascorbic acid		—	20·0	—	—
Peroxide-free ether + ascorbic acid + chromatophore pellet		—	32·5	—	—
Acetone		—	—	23·5	—

* Not measured.

ferrochelatase activity, which is always assayed in the presence of ascorbic acid (Fig. 4). Chromatophore-Tween pellet stimulated ferrochelatase activity. These observations, and particularly those showing the effect of ascorbic acid on zinc–protoporphyrin-chelatase activity, suggested the possibility of an inhibitory effect by peroxides in the ether. This effect would be observed only when assays were done in the absence of a reducing agent such as ascorbic acid. The results also suggested that at least part of the effect of chromatophore pellet might be due to some component in it reacting with the peroxides and thus protecting the enzyme.

These suggestions were confirmed by comparing activities for zinc–protoporphyrin chelatase with different concentrations of mitochondrial supernatant in the presence of both ordinary dry ether and peroxide-free ether. The activities in the presence of the latter were higher than with the former and the enzyme–activity curve was linear (Table 3). With peroxide-free ether the activity was identical with that found by using acetone and with that observed when ascorbic acid was used together with ordinary dry ether.

All further work has been done with peroxide-free ether, except where stated otherwise, and as an additional precaution ascorbic acid has been added when assaying for zinc–protoporphyrin-chelatase activity.

Effect of chromatophore-Tween pellet and of lipids on chelatase activities. Although chromatophore pellet overcame the effect of peroxides in the ether, the results in Table 3 show that even with peroxide-free ether chromatophore pellet, which had negligible chelatase activity on its own, gave a marked stimulation of zinc–protoporphyrin-chelatase activity. Chromatophore pellet also stimulated ferrochelatase activity when ether or acetone was used (Table 4).

Table 4. *Effect of chromatophore pellet on the ferrochelatase activity in mitochondrial supernatant, assayed in the presence of ether and acetone*

Supernatant was prepared from 'heavy' and 'light' mitochondria of guinea-pig liver after treating with Tween 20, as described in the text. Assay: tris, pH 8·2 (100 μmoles), protoporphyrin (50 mμmoles), $FeSO_4$ (100 mμmoles), ascorbic acid (10 μmoles), 'heavy'-mitochondrial supernatant (0·65 mg. of protein) or 'light'-mitochondrial supernatant (0·42 mg. of protein), and, where added, chromatophore pellet (0·50 mg. of protein), were mixed in 0·5 ml. of water. Acetone (0·1 ml.) or ether (0·2 ml.) was added and, after shaking, the tubes were incubated at 37°. The amount of haem formed was estimated as described by Neuberger & Tait (1964).

Source of Tween supernatant		Haem (mμmoles)
Heavy mitochondria	Ether	15·0
Heavy mitochondria + chromatophore pellet	Ether	25·0
Heavy mitochondria	Acetone	15·5
Heavy mitochondria + chromatophore pellet	Acetone	21·4
Light mitochondria	Ether	0·0
Light mitochondria + chromatophore pellet	Ether	17·9
Light mitochondria	Acetone	0·6
Light mitochondria + chromatophore pellet	Acetone	10·9

Chromatophore pellet did not lose any of its ability to stimulate chelatase activity when boiled for 3min. whereas mitochondrial supernatant was inactivated by this treatment. This finding suggested that the active factor was not a protein and this was confirmed when it was found that lipids extracted from chromatophores of chromatophore-Tween pellet and added to the assays in solution in ether stimulated chelatase activity in mitochondrial supernatant (Fig. 5). This experiment was done with ordinary ether, and while some of the effect of lipid in the zinc–protoporphyrin-chelatase assay might be to react with peroxides this cannot be the case in the ferrochelatase assay where ascorbic acid was present.

Fractionation of lipid from *R. spheroides* was performed on columns of silicic acid. Each fraction eluted from the column was evaporated to dryness and dissolved in peroxide-free ether. Ether solutions were tested for lipid phosphate, lipid composition by thin-layer chromatography, and ability to stimulate zinc–protoporphyrin-chelatase activity in mitochondrial supernatant (Table 5). It can be seen that the fraction from the first column that was active was the one eluted with methanol and which contained the bulk of the phospholipids together with the ornithine-containing lipid (Gorchein, 1964). On fractionation of these phospholipids on the second column the activity was mainly in the fraction eluted with methanol, which contained phosphatidylcholine as well as some phosphatidylethanolamine. It should be pointed out, however, that the amount of phospholipid which had to be added to obtain this stimulation was greatly in excess of the phospholipid contained in the amount of chromatophore pellet required to give the same stimulation.

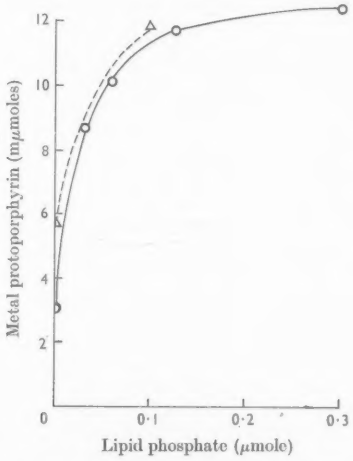

Fig. 5. Effect of chromatophore lipid on zinc–protoporphyrin-chelatase and ferrochelatase activities in mitochondrial-Tween supernatant. Tris, pH 8·4 (100 μmoles), protoporphyrin (40 mμmoles), either $ZnSO_4$ (50 mμmoles) or $FeSO_4$ (100 mμmoles) plus ascorbic acid (10 μmoles), and mitochondrial-Tween supernatant (1·17 mg. of protein) were mixed in a volume 0·5 ml. Crude lipid extract prepared from chromatophores by chloroform–methanol extraction (see the Materials and Methods section) was added to the assays in solution in ether and additional ether was added to 0·2 ml. After shaking, the samples were incubated at 37° for 90 min. and zinc protoporphyrin (○) and haem (△) were measured as described by Neuberger & Tait (1964).

Before the inhibitory effect of peroxides was appreciated a similar experiment to that reported in Table 5 was performed. In that experiment the active lipid fraction was the one containing phosphatidylglycerol and phosphatidic acid. Very little or no activity was observed in the fraction containing phosphatidylcholine.

Removal of lipid from mitochondrial-Tween supernatant by extraction at 0° with 90% (v/v) acetone reduced chelatase activities very markedly, as did similar treatment of chromatophores (cf. Table 2). Addition of chromatophore pellet or lipids extracted from chromatophores markedly stimulated both zinc–protoporphyrin-chelatase and ferrochelatase activities of these preparations.

Phosphatidic acid (prepared from egg-yolk phosphatidylcholine), but not phosphatidylcholine itself or phosphatidylethanolamine (both from egg yolk), stimulated both chelatase activities when assays were performed either in the presence or absence of ether in a manner similar to that found for chromatophores (cf. Fig. 3). Thus with mitochondrial-Tween supernatant (0·64 mg. of protein), assayed in the presence of ether, phosphatidic acid (1·0 mg.) caused an increase in zinc protoporphyrin formed from 10·0 mμmoles/hr. to 17·0 mμmoles/hr., and in the absence of ether the same amount of phosphatidic acid caused an increase from 0·0 to 4·9 mμmoles/hr. Ferrochelatase activity assayed in the presence of ether was increased from 5·1 to 7·5 mμmoles/hr. by the addition of phosphatidic acid (0·5 mg.).

Further properties of chelatases in mitochondrial extracts. A pH–activity curve for zinc–protoporphyrin chelatase obtained with mitochondrial supernatant and assaying in the presence of ether is shown in Fig. 6. There is a marked optimum at pH 8·8. This is similar to the curve found by Porra & Jones (1963*a*) for ferrochelatase in pig-liver mitochondria assayed in the absence of organic solvent.

Previous work by Mazanowska, Dancewicz & Kowalski (1962) showed that ferrochelatase activity

Table 5. *Effect of lipid fractions from* Rhodopseudomonas spheroides *on chelatase activity*

R. spheroides (2g. dry wt.), suspended in 25ml. of water, were extracted with chloroform–methanol by the technique of Bligh & Dyer (1959). This extract was evaporated to dryness and the lipid, in solution in chloroform, was applied to a column of 7g. of silicic acid. The column was eluted successively with 4vol. of chloroform, dry acetone and methanol. Each fraction was evaporated to dryness and the residue dissolved in 5ml. of peroxide-free ether. A portion (3ml.) of the fraction eluted with methanol was evaporated to dryness and applied in solution in chloroform to a column of 2g. of silicic acid. The column was eluted successively with 4 column vol. of chloroform, 5% methanol in chloroform, 15% methanol in chloroform and methanol. Each fraction was evaporated to dryness and dissolved in 3ml. of peroxide-free ether. Portions of the ether solutions were analysed for lipid phosphate and lipid composition as described in the Materials and Methods section. Samples were also tested for their ability to stimulate zinc–protoporphyrin-chelatase activity in the following assay system. In a volume of 0·5ml. of water, tris, pH 8·4 (100 μmoles), protoporphyrin (50 mμmoles), $ZnSO_4$ (50 mμmoles), guinea-pig mitochondrial supernatant (0·64mg. of protein) and ascorbic acid neutralized to pH 8·4 (10 μmoles) were mixed. Ether solutions of lipids and additional peroxide-free ether to 0·2ml. were added and, after thorough mixing, the tubes were incubated for 1hr. at 37°, after which the amount of zinc protoporphyrin formed was estimated.

Additions to assay	Lipid phosphate (μmoles/assay)	Zinc protoporphyrin (mμmoles/assay)	Main recognized components in lipid fraction
None	—	9·5	
Chromatophore pellet	0·03	14·3	
Lipid eluted from first column with chloroform	0·01	9·5	Bacteriochlorophyll, carotenoids
Acetone	0·05	9·5	Phosphatidic acid and traces of pigments
Methanol	1·15	11·5	Phosphatidic acid, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine and ornithine lipid
Lipid eluted from second column with chloroform	0·03	9·4	None
5% methanol in chloroform	0·16	9·8	Phosphatidic acid, phosphatidylglycerol, ornithine lipid
15% methanol in chloroform	0·51	11·0	Phosphatidylglycerol, ornithine lipid, phosphatidylethanolamine
Methanol	0·62	14·1	Phosphatidylethanolamine, phosphatidylcholine

in mitochondria from a number of animal tissues was markedly inhibited by serum, and that the inhibitor appeared in fractions V, VI/2 and VI/3 of Cohn *et al.* (1946), which consist mainly of albumin and α-globulin. The assays of enzyme activity in that work were performed in the absence of organic solvent. When bovine plasma albumin was used instead of serum, zinc–protoporphyrin-chelatase and ferrochelatase activities were markedly inhibited, but only if assays were done in the absence of organic solvent. Thus 3mg. of albumin per assay in the presence of 1·5mg. of mitochondrial protein per assay inhibited the ferrochelatase by 100% and the zinc–protoporphyrin chelatase by 85%, whereas 1·5mg. of albumin under the same conditions gave about 50% inhibition of ferrochelatase. In the presence of an optimum amount of ether, 3mg. of albumin per assay under the above conditions gave no inhibition.

Attempts to purify the enzyme(s) responsible for the chelatase activities by treating mitochondrial supernatant with different amounts of ammonium sulphate were only partly successful, giving usually a twofold purification on a protein basis in the fraction precipitating between 20 and 40% saturation.

Mitochondrial supernatant, before or after dialysis or after fractionating with ammonium sulphate, was chromatographed on columns of carboxymethylcellulose and diethylaminoethylcellulose. Under a variety of conditions only two major protein peaks were eluted from each column, both of which contained chelatase activity, but the specific activity was not increased. Chromatography on columns of Sephadex G-50, G-100 and G-200 gave a protein peak which was eluted with the front and which contained all the chelatase activity.

DISCUSSION

Comparison of the non-enzymic and enzymic incorporation of zinc and iron. The reason for the increase in the rate of the non-enzymic formation of zinc protoporphyrin at high pH values is not known, but it may be related to a change in the

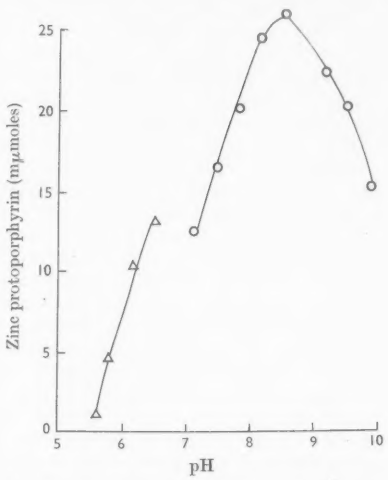

Fig. 6. pH–activity curve for mitochondrial zinc–protoporphyrin-chelatase activity. Maleate buffer (30 μmoles) (△) or tris buffer (100 μmoles) (○), pH stated, protoporphyrin (44 μmoles), $ZnSO_4$ (40 mμmoles) and guinea-pig-liver mitochondrial-Tween supernatant (1·33 mg. of protein) were mixed in a volume 0·5 ml. Ether (0·2 ml.) was added and, after thorough mixing, the samples were incubated at 37° for 90 min. Zinc protoporphyrin was measured as described by Neuberger & Tait (1964).

solubility or the state of ionization of the protoporphyrin or to a change in the state of the zinc. The stimulation by sodium lauryl sulphate of the formation of complexes between protoporphyrin dimethyl ester and a number of metal ions was described by Lowe & Phillips (1961), who considered that the detergent micelle reacted with protoporphyrin dimethyl ester, thereby rendering it soluble, and that metal ions were attracted to the anionic micelle–water interface, where reaction took place.

A comparison of the properties of these two systems forming zinc protoporphyrin with the reaction occurring in the presence of chromatophores and mitochondria shows that they are different in type and that the chromatophores and mitochondria cannot be considered simply as facilitating the reaction by raising the pH locally or by acting as anionic detergents. At high pH values and in the presence of sodium lauryl sulphate, organic solvents did not stimulate the rate of zinc protoporphyrin formation, whereas they did in the presence of chromatophores and mitochondria. In the non-enzymic reactions the rates increased up to pH 11 (Fig. 1), whereas with both chromatophores (Neuberger & Tait, 1964) and mitochondria (Fig. 6) the rates increased with pH up to 8·2 and 8·8 respectively and above these pH values the rates decreased.

Some evidence, such as values for the heat of activation of the reaction and the instability of the active factor to heat, indicated that the formation of zinc protoporphyrin and haem catalysed by chromatophores and mitochondria was enzymic in nature (Neuberger & Tait, 1964). Additional evidence, namely that of the reduction of activity by treatment with trypsin or chymotrypsin, is given in this paper. Although non-enzymic formation of haem was not detected in the present experiments, this was probably due to the insensitivity of the assay method used. Non-enzymic formation of haem was detected in previous work (cf. Granick & Mauzerall, 1958; Lowe & Phillips, 1961), but the rate of this reaction is very much lower than that catalysed by biological extracts (Labbe, 1959).

Probable identity of zinc–protoporphyrin-chelatase and ferrochelatase. In the experiments described in this and the previous paper (Neuberger & Tait, 1964) addition of ether caused an increase of zinc–protoporphyrin-chelatase and ferrochelatase activities of mitochondria and chromatophores. However, in the early experiments with mitochondria, when no special precautions were taken to free the ether of peroxides, differences between some of the properties of the two activities were noted. These differences were not observed when the assays were done in the presence of acetone, and were also not observed when ascorbic acid was added to the zinc–protoporphyrin-chelatase assay or when peroxide-free ether was used. Indeed it became apparent that peroxide-free ether and acetone had similar effects on both zinc–protoporphyrin-chelatase and ferrochelatase activities in both chromatophores and mitochondria. In view of the probable involvement of lipid in chelatase activity, evidence for which is discussed below, it is possible that peroxides exert their inhibitory effect by oxidizing this lipid, although they may also inhibit by oxidizing thiol groups in the protein. Conversely, reducing agents probably prevent the oxidation of the lipid as well as protect essential thiol groups and keep the ferrous iron reduced, as suggested by Labbe & Hubbard (1960) and Porra & Jones (1963*a*).

It appears that zinc–protoporphyrin chelatase and ferrochelatase behave very similarly under most of the conditions described in this paper, suggesting that one enzyme is responsible for both activities. Some of the earlier results reported by Neuberger & Tait (1964), which had suggested that different enzymes might be involved, are probably explained by the presence of a reducing agent, usually ascorbic acid, in the ferrochelatase assay but not in the zinc–protoporphyrin-chelatase assay: thus the effects of peroxides were eliminated in one assay but not in the other.

Involvement of lipid in chelatase action. A role of lipid in chelatase action was suggested by a number of findings. The chelatase in mitochondrial supernatant was stimulated in the presence of ether or acetone by chromatophore pellet, or a chloroform–methanol extract from chromatophores. Removal of lipid by preparation of acetone-dried powders or extraction of chromatophores or mitochondria with 90% (v/v) acetone reduced activity in the presence of ether and acetone. Activity could be partially restored by adding lipid or an extract containing lipid (e.g. chromatophore pellet). Phosphatidic acid, added as an aqueous emulsion or in ether solution, stimulated chelatase activity in chromatophores and in mitochondria. Albumin, or serum fractions containing albumin, reduced chelatase activity, but only in the absence of organic solvents. It may be that this action is due to the binding of lipids by the albumin. That the inhibitory effect is not due to the binding of metals by albumin was suggested by the fact that the inhibition was not reversed by excess of Fe^{2+} ions (Mazanowska *et al.* 1962).

While this work was in progress a similar finding of lipid involvement in ferrochelatase activity was reported by Yoshikawa & Yoneyama (1964). They showed that the activity of a soluble enzyme system from duck erythrocyte stroma was increased by the addition of an aqueous emulsion of phospholipid extracted from the same source.

Phospholipids, which have been shown to play a role in the function of a number of enzymes, particularly those bound to particles such as mitochondria (Asano, Kaneshiro & Brodie, 1965), could act in either of two ways: by aiding the binding of substrates to the enzyme, or by directly affecting the enzyme structure and therefore its function. Although the first explanation may apply to enzymes acting on compounds which are not water-soluble, it is not satisfactory for enzymes such as D-β-hydroxybutyrate dehydrogenase (EC 1.1.1.30; Jurtshuk, Sekuzu & Green, 1963) and malate–oxygen oxidoreductase (EC 1.1.3.3) using phenazine as electron acceptor (Tobari, 1964) whose substrates are water-soluble. In these enzymes it is suggested (Tobari, 1964) that phospholipid is an integral part of the enzyme complex, its presence being required for the formation of an 'active centre' in the enzyme molecule; such complexes are probably the natural form of these enzymes in intact subcellular particles. Very recently it has been shown by Pesch & Peterson (1965) that enzymic removal of phospholipid from mitochondrial $NADPH_2$–NAD oxidoreductase (EC 1.6.1.1) changes the substrate specificity of the system. It would seem likely that this is caused by a modification of the conformation of the protein, perhaps especially at its active site.

The active lipid extracted from *R. spheroides* appears to be phosphatidylcholine, but, as can be seen from Table 5, the amounts of lipid required to show stimulation of activity were much greater than the lipid contained in the amount of chromatophore pellet which gave the same degree of stimulation. This may mean that the lipid is unstable to the manipulations involved in extraction and fractionation or that when added in ether solution its ability to stimulate activity is much poorer than when present in its native state. Phosphatidylcholine prepared from egg yolk had no effect on chelatase activity, whereas phosphatidic acid prepared by enzymic hydrolysis of this phosphatidylcholine was active both in the presence and absence of ether. These observations might suggest that the two phospholipids act in different ways. Ward & Fantl (1963) reported that a number of hydrophilic cations could be transferred from an aqueous to a lipophilic phase by shaking a solution containing the cation with an ether solution of phosphatidic acid. This observation might suggest that phosphatidic acid, which is present in small amounts in chromatophores and also in mitochondria (Wajtczak, Wlodawer & Zborowski, 1963), binds Zn^{2+} and Fe^{2+} ions, and in this way facilitates chelatase reactions. Phosphatidic acid, as an aqueous emulsion, or in ether solution, protected the chromatophore system from inhibition by excess of Zn^{2+} ions, which might also be explained by its capacity to form a zinc salt. The action of phosphatidylcholine, on the other hand, might be due to its ability to react with the enzyme and alter its activity. For this the fatty acid composition may be important. In this connexion, Jurtshuk *et al.* (1963) found that only phosphatidylcholine which contained unsaturated fatty acids acted as cofactor for D-β-hydroxybutyrate dehydrogenase.

Action of organic solvents. After removal of lipid from chromatophores or mitochondria the activity dropped markedly when assayed in the presence of organic solvents, but could be restored by adding back lipid or an extract containing lipid (e.g. chromatophore-Tween pellet). It is therefore reasonable to assume that the organic solvents act in some way in conjunction with the lipid present in the chromatophores or mitochondria so as to alter the rate of the enzyme reaction. At present there is no evidence on the exact way in which this occurs.

Organic solvents may facilitate binding between the enzyme and the substrate, as has been suggested by Kates (1953, 1956, 1957) to account for their stimulatory effect on the phospholipase D (EC 3.1.4.4) of chloroplasts. The effect of ether on phospholipases A (EC 3.1.1.4) and B (EC 3.1.1.5), whose action produces fatty acids, has been noted by Hanahan (1952), Hanahan, Rodbell & Turner

(1954), Magee & Thompson (1960) and Dawson (1963). These workers considered the action of ether to involve first the breakdown of larger micelles of phosphatidylcholine to ones of smaller size, thereby increasing the availability of the substrate to the enzyme, and secondly the removal from the micelle–enzyme interphase of the fatty acids, whose accumulation would inhibit the reaction. Although protoporphyrin in water buffered at pH 8·4 is not in true solution (cf. Porra & Jones, 1963*b*), it is unlikely that the organic solvents act by changing the physical state of protoporphyrin since they have no effect on the non-enzymic formation of zinc protoporphyrin. Yoshikawa & Yoneyama (1964) reported that incubation of δ-aminolaevulic acid with haemolysates of duck erythrocytes yielded protoporphyrin, the bulk of which appeared in some way to be attached to the phospholipid of the stroma. They also showed that the 'lipid-bound' protoporphyrin was more readily converted into haem than was free protoporphyrin. Thus organic solvents may facilitate the combination of protoporphyrin with the phospholipid of chromatophores or of mitochondria and so aid the formation of metal complexes.

The effect of phosphatidic acid on chelatase activity and the possible mechanism by which this occurs was discussed above. It might be suggested that the ability of phosphatidic acid to form salts with zinc and iron is facilitated in the presence of organic solvents and in this way the rate of production of zinc protoporphyrin and haem is increased.

In conclusion it would appear that the chromatophore and mitochondrial enzymes responsible for chelation of zinc and iron with protoporphyrin are present in the native state in close association with phospholipid. Whether the phospholipid acts by combining with the enzyme in such a way as to produce favourable enzymic conformation or whether it acts by facilitating combination of the two substrates with the enzyme is still unknown. The effect of organic solvents will probably be understood when the exact mechanism of action of phospholipids is explained.

A. M. M. held a Fellowship of the International Atomic Energy Agency during this work.

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The Distribution of Free Amino Acids in Subcellular Fractions of Guinea-Pig Brain

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(*Received* 1 *June* 1965)

1. The free amino acids of homogenates of guinea-pig brain in 0·32M-sucrose and of subcellular fractions derived therefrom have been estimated by the method of Moore & Stein. 2. Seven amino acids together accounted for over 80% of the free amino compounds; these are, in decreasing order of abundance: glutamate, aspartate, γ-aminobutyrate, glycine, serine, alanine and threonine. In addition, there are appreciable quantities of amide (presumably glutamine). 3. Control experiments showed that the pattern of free amino acid occurrence in sucrose homogenates was similar to that of brains of animals killed by freezing in liquid nitrogen and extracted immediately without thawing. 4. The subcellular distribution of the amino acids resembled that of soluble cytoplasmic markers; there was no specific localization in a fraction rich in isolated presynaptic nerve terminals of amino acids capable of exciting or depressing central neurones. 5. The significance of the results is discussed in relation to the possible role of centrally active amino acids as transmitters.

Two amino acids present in uniquely high concentration in brain tissues, glutamate and γ-aminobutyrate, have attracted attention as possible synaptic transmitter substances because of their ability, in very small amounts, to stimulate or depress cortical and other central neurones. As little as 10^{-15}moles of glutamate, if applied iontophoretically through a micropipette in close proximity to a cortical neurone, will cause it to discharge a train of electrical impulses, and similar amounts of γ-aminobutyrate will depress neuronal activity (Krnjević & Phillis, 1963; Krnjević, 1965).

For this reason we have for some time been studying the subcellular distribution of glutamate, γ-aminobutyrate and other free amino acids of brain tissue by using an automatic amino acid analyser and subcellular fractionation techniques developed in this Laboratory for the study of bound acetylcholine and other putative central transmitters (reviewed by Whittaker, 1965). When brain tissue is homogenized under carefully controlled conditions, a considerable proportion of the nerve endings survive homogenization and are torn away from their attachments. The free nerve endings, for which the term 'synaptosomes' has been proposed (Whittaker, Michaelson & Kirkland, 1964), may be separated as a distinct fraction by differential and density-gradient centrifuging. This fraction may be expected to be rich in transmitter substances; and it is, in fact, found to contain most of the bound acetylcholine, noradrenaline and hydroxytryptamine of the tissue. It also provides a starting point for the preparation of component structures of the nerve endings such as synaptic vesicles, external membranes, intraterminal mitochondria and neuronal cytoplasm. Other fractions obtained from the original homogenate contain nuclei, myelin fragments, mitochondria, microsomes and cell sap. The analysis of all these fractions can thus provide much information about the distribution of substances within morphologically defined subcellular compartments of nervous tissue.

A preliminary account of our work on amino acid distribution was given by Whittaker (1963). During the course of this work, column-chromatographic analyses of the free amino acids of bee and dog brain have been presented by Frontali (1962) and Tews, Carter, Roa & Stone (1963) respectively. Earlier work has been reviewed by the latter authors. The subcellular distribution of glutamate and γ-aminobutyrate in rat and mouse brain has been studied by Ryall (1964) and Weinstein, Roberts & Kakefuda (1963) by using fractionation procedures based on earlier work from this Laboratory (Hebb & Whittaker, 1958; Whittaker, 1959).

METHODS

Preparation of brain fractions

Two series of experiments were carried out, with whole brain and cortex respectively. The general conditions were similar to those described by Gray & Whittaker (1962), and were such as to provide a maximum yield of synaptosomes (Whittaker & Dowe, 1965). Homogenization was carried out in a glass-and-Perspex homogenizer of the type described by Aldridge, Emery & Street (1960) with a clearance (on diameter) of 0·25 mm. and a rotational speed of 840 rev./min. All operations were carried out at 0–4° in a cold laboratory with glass-distilled water and analytical-grade reagents.

Primary fractions. Unless otherwise stated, three or four guinea-pig brains (the whole brain rostral to the superior colliculi; total weight 8–12 g.) were homogenized in ice-cold 0·32 M-sucrose to give a final tissue concentration of 10% (w/v). In some experiments the homogenate was centrifuged immediately, or at various times after preparation, at 100000*g* for 60 min. to give a total particulate preparation (*P*) and a high-speed supernatant (*S*). In other experiments it was separated into nuclear (P_1), crude mitochondrial (P_2), microsomal (P_3) and supernatant (S_3) fractions as described by Gray & Whittaker (1962). The particulate fractions were sedimented by centrifuging the homogenate, or supernatant from the previous spin, at 1000*g* for 11 min. (P_1 fraction), 17600*g* for 60 min. (P_2 fraction) and 100000*g* for 60 min. (P_3 fraction). The P_1 fraction was twice washed by resuspension in 0·32 M-sucrose and recentrifuging at 1000*g* for 11 min.; the washings were added to the initial supernatant before centrifuging the latter to give fraction P_2. Pellets were resuspended in 0·32 M-sucrose where necessary for analysis.

The P_2 fraction was separated into three subfractions containing mainly myelin (fraction *A*), synaptosomes (fraction *B*) and mitochondria (fraction *C*) by means of a two-step discontinuous density gradient made up of equal volumes of 0·8 M- and 1·2 M-sucrose as described by Gray & Whittaker (1962). Separation was achieved in the SW 25 head of the Spinco model L preparative ultracentrifuge after 2 hr. at 53500*g*. Fraction *A* was the material floating on 0·8 M-sucrose, fraction *B* that floating between 0·8 M- and 1·2 M-sucrose and fraction *C* that sedimenting below 1·2 M-sucrose.

For preparing subfractions of disrupted synaptosomes (see below) the P_1 fraction was not washed and the P_2 fraction was sedimented at 10000*g* for 20 min. This diminished the yield of synaptosomes but speeded preparation and cut down microsomal contamination. Similar fractions were prepared from guinea-pig cortical tissue.

Subfractions of disrupted synaptosomes. Fractions containing the soluble cytoplasmic constituents of synaptosomes (fraction *O*), synaptic vesicles (fraction *D*), microsomes (fraction *E*), larger (external) membrane fragments (fractions *F* and *G*), incompletely disrupted synaptosomes (fraction *H*) and mitochondria (fraction *I*) were prepared from the supernatant (W_s) of a P_2 fraction from whole brain after resuspension in water and removal of larger fragments (mostly myelin fragments and large mitochondria) at 10000*g* for 20 min. as described by Whittaker *et al.* (1964). Similar fractions were prepared from cortical synaptosomes by 'method B' of Eichberg, Whittaker & Dawson (1964) (see also Whittaker & Sheridan, 1965). In this procedure, the cortical P_2 fraction described above was washed by resuspension in sucrose, resedimented at 10000*g* for 30 min. and then disrupted by suspension in water (2 ml./g. of original tissue). The suspension was layered (5 ml./tube) on top of a continuous sucrose density gradient ranging from 0·4 M-sucrose at the top to 0·6 M-sucrose at the bottom (15 ml./tube), which had in turn been formed on top of a layer of 1·6 M-sucrose (5 ml./tube) in a Lusteroid tube of the Spinco SW 25 head. After centrifuging at 53500*g* for 2 hr., a clear top layer (*O*) containing soluble cytoplasmic components had formed above a hazy band (*D*) of synaptic vesicles; below this, floating between 0·6 M- and 1·6 M-sucrose, was a dense layer (*J*) containing partially disrupted synaptosomes, membrane fragments and mitochondria. This fraction corresponded in density and composition to fractions *E–I* of the whole-brain preparations. The tube was sliced to separate the clear, hazy and dense layers. In some experiments, the *D* layer was diluted with an equal volume of water and centrifuged at 100000*g* for 60 min. to give an almost clear supernatant (D_s) and a pellet (D_p) of closely packed synaptic vesicles.

Frozen brain extracts. To compare the amino acid content of sucrose homogenates with that of brain extracts prepared under conditions in which post-mortem changes in the concentration of labile constituents are believed to be minimal, small guinea pigs (weighing less than 200 g.) were killed by immersion in liquid N_2 and the frozen brains removed from the skull by means of a cold chisel and bone forceps. The brain was quickly powdered while still frozen in a chilled mortar and extracted with ethanolic 12·5 mN-HCl (4 vol. diluted with 1 vol. of water; 100 ml. of diluted extractant/g. of tissue).

Extraction and analysis of amino acids

Amino acids were extracted from brain fractions by the addition of 4 vol. of ethanolic 12·5 mN-HCl. Particulate material, if still present, was removed by centrifuging the suspension at 10000*g* for 20 min. and the supernatant (or original solution) reduced in volume *in vacuo* to about 10 ml. in a rotary evaporator at 35°. To avoid interference by sucrose and electrolytes in subsequent chromatography, the amino acids were adsorbed from the extract on to the acidic ion-exchange resin, Zeo-Karb 225 (15–30 mesh; The Permutit Co. Ltd., London) and eluted with aq. NH_3 as described below. The Zeo-Karb 225 was prepared for use by heating it (0·45 kg.) with three successive amounts (1 l.) of N-NaOH at 100° for 2 hr. The resin was then washed thoroughly with water, converted into the H^+ form with N-HCl and finally again washed with water.

Adsorption and elution of amino acids from fractions. Two polythene bottles, each containing 4 g. of resin, were used for each extract. The resin samples were first washed by shaking with water, which was discarded. The fraction was poured into bottle 1 of the pair, shaken mechanically for 10 min., the resin allowed to settle and the supernatant transferred to bottle 2. An equal quantity of water was added to bottle 1. The bottles were then shaken for a further 10 min. The supernatant in bottle 2 was discarded, that from bottle 1 transferred to bottle 2 and a further quantity of water added to bottle 1. The shaking and transfer procedure was repeated. Finally, the supernatant from bottle 2 was again discarded and that of bottle 1 was transferred to bottle 2, which was shaken for 10 min., after which the supernatant was discarded.

The amino acids were eluted from the resin by means of

aq. 5N-NH_3 (10–20ml.) added to each bottle. After shaking for 10min. the supernatants were collected and elution was repeated with a further 10–20ml. of aq. 5N-NH_3. The combined supernatants were reduced in volume on the rotary evaporator and made up to 5ml. Samples were taken for amino acid analysis by column chromatography as described below. For total amino nitrogen estimations by the ninhydrin method the extracts were freed from any traces of NH_3 still present by adding an equal volume of 10% (w/v) potassium metaborate and storing them over conc. H_2SO_4 and P_2O_5 in a desiccator for 12–15hr. under reduced pressure. They were then diluted 1:10 before analysis.

Ninhydrin-positive substances in column-chromatographic fractions were similarly extracted for subsequent paper chromatography, except that 40g. quantities of resin were used per bottle.

Estimation of carboxyl amino nitrogen. This was carried out on the extracts manometrically by using the chloramine-T method of Gerok & Waller (1956).

Estimation of total amino nitrogen by ninhydrin. This was carried out essentially as described by Chibnall, Mangan & Rees (1958). Diluted extract (1ml.), freshly prepared KCN–acetate buffer (made by mixing 23ml. of 4M-sodium acetate buffer, pH5·5, 75ml. of 2-methoxyethanol and 2ml. of 10mM-KCN) (1ml.) and 10% (w/v) ninhydrin (0·2ml.) were heated at 100° for 15min. in capped tubes, cooled and diluted to 10ml. with ethanol, and the extinctions at 570mμ compared with those of (*a*) standard solutions of glutamic acid containing 1–6μg. of N/ml. and (*b*) a resin blank obtained by shaking resin with water and eluting any adsorbed substances with aq. NH_3. Since the extinctions of the ninhydrin complex with different amino compounds vary, the method cannot claim to be very accurate, but it was useful in deciding how much of a fraction was needed for chromatography and to get an approximate idea of how total amino nitrogen was distributed in the various fractions.

Analysis of constituent amino acids of extracts by column chromatography. The constituent amino acids of the extract were analysed by chromatography on jacketed columns of sulphonated polystyrene resin by using an automatic analyser to record the concentration of ninhydrin-positive material in the effluent. The analyser made use of a sampling and mixing device based on two motor-driven syringes. A complete analysis required about 7μg.atoms of amino nitrogen. Initially, neutral and acidic amino acids and basic amino acids were analysed separately, the former by the two-buffer system of Spackman, Stein & Moore (1958) with a 1cm.×150cm. column and 0·2M-sodium citrate buffer, pH3·25, at 30° followed by 0·2M-sodium citrate buffer, pH4·25, at 50°, and the latter on a 1cm.×50cm. column with 0·38M-sodium citrate buffer, pH4·26, at 30° and 50°. Later a simpler procedure was developed by using a 1·5cm.×60cm. column of resin beads of uniform (18μ, S.D. $\pm$4μ) diameter (Hill & Mangan, 1964) and gradient elution. After an initial elution with 0·2M-sodium citrate buffer, pH3·25, at 30°, elution with 0·2M-sodium citrate buffer, pH4·25, at 50° was commenced and the ionic strength and pH of the eluting fluid were continuously raised by the addition of 0·33M-trisodium citrate to the buffer reservoir. The amount of each component was estimated by integration of the area under the peaks by using the width-and-height method of Spackman *et al.* (1958).

Table 1. *Recovery of amino acids from a mixture*

Amino acids in mixture	Concn. in mixture (μg.atoms of N/ml.)	Amount recovered (% of original amount)
Aspartate	1·22	112
Threonine	1·89	98
Serine	1·26	98
Glutamate	0·89	128
Glycine	3·37	98
Alanine	1·00	94
γ-Aminobutyrate	1·80	80
Lysine	1·79	100
Total	13·17	102
Total amino N (by ninhydrin)	10·17	77

Combined paper chromatography and paper electrophoresis of fractions separated by column chromatography. The compounds present in column-chromatographic peaks were adsorbed on to resin and eluted as described above. They were then submitted to combined paper chromatography and paper electrophoresis as described by Dawson, Hemington & Davenport (1963), by using chromatography in a phenol–acetic acid–ethanol–water mixture in one direction and ionophoresis at pH3·6 at right-angles to this. Ninhydrin-positive and phosphorus-containing substances were localized by appropriate sprays.

Recoveries of amino acids. Table 1 shows that satisfactory recoveries are obtained when a known mixture of amino acids in concentrations comparable with those found in the brain extracts were put through the analytical procedure just described.

Estimation of total nitrogen. This was carried out by a micro-Kjeldahl procedure (Chibnall, Rees & Williams, 1943).

Other analytical procedures

Estimation of total phosphorus. Total phosphorus in column effluents was determined by the method of Fiske & Subbarow (1925) after digestion with $HClO_4$. Samples of the fractions (2ml.) were digested for 25min. with 72% (w/v) $HClO_4$ (0·9ml.), cooled and diluted with water (8·4ml.); 5% (w/v) ammonium molybdate (0·5ml.) and aminonaphtholsulphonic acid reagent (0·4ml.) were added with shaking, and after 20min. the extinction at 660mμ was compared with that of suitable standards.

Estimation of lactate dehydrogenase. The enzyme was estimated spectrophotometrically on a Unicam SP.700 recording spectrophotometer as described by Johnson (1960) by following the fall in extinction at 340mμ that accompanies the transfer of hydrogen from $NADH_2$ to pyruvate.

Estimation of potassium. This was carried out in a flame photometer (Baird Atomic model KY) on samples suitably diluted with water, with lithium as an internal standard.

Estimation of acetylcholine. This was carried out by using the dorsal muscle of the leech and the micro-assay procedure of Szerb (1961) as modified by Whittaker *et al.* (1964).

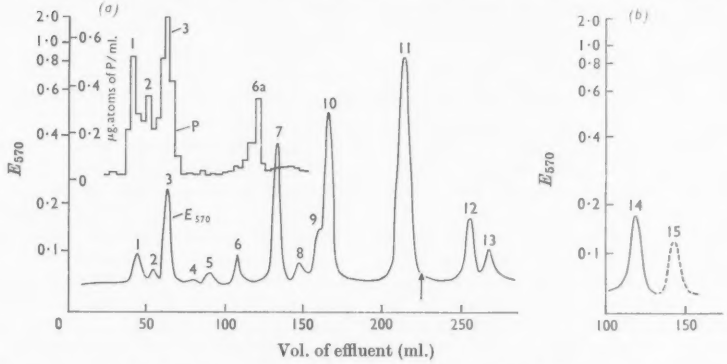

Fig. 1. Chromatograms of resin extract of homogenate from 0·28g. of guinea-pig forebrain: (*a*) acidic and neutral amino acids; (*b*) basic amino acids. Continuous tracing, ninhydrin colour; histogram, total phosphorus. For details see the text. The arrow in (*a*) indicates the change from buffer at pH3·25 and 30° to that at pH4·25 and 50°. In (*b*), the portion of chromatogram shown was obtained at 30°; no further major components emerged when the temperature was raised to 50°. Identifications: 1–3 and 6, see the text; 4, taurine; 5, urea; 7, aspartate; 8, threonine; 9, serine; 10, asparagine+glutamine (not resolved); 11, glutamate; 12, glycine; 13, alanine; 14, γ-aminobutyrate; 15, NH_3 (derived from the resin extract).

RESULTS

Amino acids of brain homogenates and fractions

Identification of components. Fig. 1 shows a chromatogram obtained from an extract of a guinea-pig forebrain homogenate. Fifteen ninhydrin-positive peaks were obtained. With the exception of peaks 1–3 and 6 these were readily identified by means of runs with known substances (see the legend to Fig. 1). Peaks 1, 2 and 3 were approximately in the positions expected for phosphorylserine, glycerylphosphorylethanolamine and phosphorylethanolamine respectively. Phosphorus analyses (upper tracing in Fig. 1*a*) showed that phosphorus was indeed present in the fractions corresponding to these peaks, but the molar P/NH_2 ratios varied across the peaks and were greater than unity. A fourth phosphorus-containing ninhydrin-negative component (peak 6a) appearing between peaks 6 and 7 was not further investigated.

Complex nature of material in peaks 1–3. The material in the fractions corresponding to peaks 1–3 and peak 6 was concentrated by adsorption on to and elution from resin as described in the Methods section and submitted to combined chromatography and electrophoresis on paper by the method of Dawson *et al.* (1963). The materials in peaks 1–3 were found to be complex mixtures. The phosphorus-containing material in peak 1 was mainly glycerylphosphorylcholine (75%) and glycerylphosphorylethanolamine (17%), and there were present in addition seven unidentified non-phosphorus-containing ninhydrin-positive substances. The phosphorus compounds of peak 2 consisted almost entirely of glycerylphosphorylcholine and glycerylphosphorylethanolamine in approximately equal proportions, together with traces of phosphorylethanolamine and six other ninhydrin-positive substances all different from those in peak 1. Peak 3 consisted mainly of phosphorylcholine together with inorganic phosphate and five non-phosphorus-containing ninhydrin-positive substances. The ninhydrin-positive substances in these fractions are presumably acidic peptides; the heterogeneity of the fractions is not surprising in view of the poor resolving power of the column in this region.

Identification of peak 6. In contrast with peaks 1–3, the material in peak 6 appeared to consist of one main ninhydrin-positive compound. On keeping in aqueous solution it slowly underwent spontaneous hydrolysis with the release of glutamate; this suggested the presence of a γ-glutamyl residue. On refluxing for 24hr. in a mixture containing hydrochloric acid (5N) and formic acid (10N) it was hydrolysed to glutamate with smaller amounts of aspartate, serine and glycine. This suggested that the main component might be γ-L-glutamyl-L-glutamate, recently isolated from ox brain by Kakimoto, Nakajima, Kanazawa, Takesada & Sano (1964) and appearing on their chromatograms in a similar position to our peak 6, 19ml. after urea. A sample of synthetic glutamylglutamate kindly supplied by Dr Y. Kakimoto had the same retention

volume as peak 6 in our system. No indication was obtained for the presence of γ-glutamylglutamine, also isolated by Kakimoto *et al.* (1964).

Unidentified peaks labelled 'peptide' are seen in positions corresponding to our peak 6 in the chromatograms of hen-brain and bee-brain extracts published by Baker & Porcellati (1959) and Frontali (1962) respectively. Frontali's material yielded aspartate, serine, alanine, glycine and histidine as well as glutamate on hydrolysis.

Amino acids. In addition to the four peaks described above there were peaks corresponding to urea (no. 5), ammonia (derived from the resin eluate in the initial extraction procedure, no. 15) and nine amino acids, the most abundant of which (Table 2) was glutamate, followed, in decreasing concentration, by aspartate, γ-aminobutyrate, glutamine, glycine, serine, alanine, threonine and taurine. The glutamine peak may have contained some asparagine, since the system used could not resolve these two amides; however, the amount of asparagine in brain is known to be very low (Krebs, 1950). When larger amounts of material were chromatographed a number of other amino acids could be detected as minor components. The concentrations found were similar to those reported by Tews *et al.* (1963) for dog brain except that those of taurine and amides were lower and those of γ-aminobutyrate, glycine, serine and alanine were somewhat higher.

Comparison with frozen brain. To determine the extent of any post-mortem changes in the concentration of free amino acids occurring during dissection and homogenization an analysis was made of extracts of brains of animals killed by immersion in liquid nitrogen. This method of extraction gives much higher values for ATP than homogenization in sucrose (Nyman & Whittaker, 1963) but not for other labile constituents such as acetylcholine (V. P. Whittaker & G. H. C. Dowe, unpublished work), which must therefore be protected more effectively before and during homogenization from enzymic destruction. A comparison of the results obtained with frozen brain and homogenates (Table 2) shows that the former contains about 40% more amino nitrogen, but the pattern of amino acid composition is similar in both preparations. The younger ages of the animals used in the experiments with frozen brain may account for part of the difference.

Table 2. *Amino acid analyses of frozen and homogenized guinea-pig forebrain*

The frozen brains were taken from young animals (wt. 200g.); the sucrose homogenate was of brains from adult animals (wt. 450–600g.).

Amino acid	Concn. (μmoles/g. of tissue) Frozen brain	Sucrose homogenate
Non-P-containing amino compounds		
Glutamate	10·00	7·59
Glutamine	4·33	1·27
γ-Aminobutyrate	3·28	1·94
Aspartate	2·56	2·40
Glycine	0·90	1·00
Valine	0·62	Trace
Serine	0·46	0·88
Alanine	0·30	0·63
Threonine	0·29	0·23
Peak 6	0·23*	0·28
Isoleucine	0·08	Trace
Taurine	Trace	Trace
Urea	Trace	Trace
Total non-P-containing acids	23·10	16·22
P-containing peaks		
Peak 3	1·68*	0·99
Peak 1	0·42*	0·31
Peak 2	0·33*	0·15
Total	2·43*	1·45

* Leucine equivalent.

Distribution of amino acids in primary subcellular fractions

Table 3 shows the distribution in subcellular fractions of forebrain of the eight most abundant amino acids. Also included are the peptide in peak 6, the mixtures of ninhydrin-positive substances present in peaks 1–3, total nitrogen, lactate dehydrogenase and potassium.

Except for γ-aminobutyrate, the recoveries of the amino acids in the various fractions were close to 100%; γ-aminobutyrate showed recoveries of greater than 100% (124 and 126%) in the two experiments carried out, and glutamate was the only amino acid to show, on average, a recovery appreciably less than 100%. It seems possible that some decarboxylation of glutamate to γ-aminobutyrate may have occurred during the separation.

The distribution of the various amino acids in the different fractions is fairly uniform. Between 69% (serine) and 82% (threonine) are recovered in the high-speed supernatant fraction, S_3. The remainder is distributed through the other fractions, the B fraction with two exceptions accounting for the next highest proportion. It is likely that the amino acid content of the particulate fractions may be accounted for by the presence in them of cytoplasm within unbroken cells (P_1), myelin-rich axonal fragments (P_1 and A) and synaptosomes (B). This is borne out by the similarity of their distribution pattern to that of lactate dehydrogenase

Table 3. *Distribution of amino acids, total nitrogen, lactate dehydrogenase and potassium in subcellular fractions of guinea-pig brain*

The results given are the means of two (non-phosphorus-containing amino compounds, total nitrogen), three (phosphorus-containing compounds, potassium) or four experiments (lactate dehydrogenase). For nomenclature of fractions see the text.

Compound	Concn. (μmoles/g. of tissue)	Recovery in fractions (% of homogenate)	Distribution in fractions (% of amount recovered)						Ratio of S_3 to total particulate
			P_1	A	B	C	P_3	S_3	
Non-P-containing amino compounds									
Glutamate	7·59	86	6	7	11	1	3	72	2·51
Aspartate	2·40	107	6	9	8	1	2	74	2·84
γ-Aminobutyrate	1·94	125	6	5	8	0	3	78	3·54
Glutamine	1·27	96	5	7	9	1	3	74	2·84
Glycine	1·00	98	6	9	10	2	2	71	2·45
Serine	0·88	93	5	9	12	2	3	69	2·22
Alanine	0·63	92	7	10	9	2	2	70	2·34
Peak 6	0·28*	97	5	4	11	1	2	77	3·35
Threonine	0·23	100	4	3	11	0	0	82	4·55
P-containing peaks									
Peak 3	0·99*	90	6	5	10	1	2	76	3·17
Peak 1	0·31*	116	17	6	14	4	4	55	1·22
Peak 2	0·15*	183	12	22	38	5	7	16	0·19
Other									
Total nitrogen	1·07†	100	29	11	18	7	8	26	0·35
Lactate dehydrogenase	47·6‡	90	10	6	9	2	3	70	2·34
Potassium	0·10†	105	9	6	10	1	2	73	2·70

* Leucine equivalent.
† mg.atoms/g.
‡ μmoles of $NADH_2$ disappearing/min./g. of tissue.

Table 4. *Relative concentrations of amino acids, lactate dehydrogenase and potassium in brain fractions*

For nomenclature of fractions see the text.

Fraction	Relative concn.		
	Amino acids	Lactate dehydrogenase	Potassium
P_1	0·20	0·34	0·31
A	0·67	0·54	0·54
B	0·55	0·50	0·56
C	0·15	0·29	0·14
P_3	0·27	0·37	0·40
S_3	2·83	2·69	2·81

(Table 3, l. 14) and potassium (Table 3, l. 15), both of which may be regarded as soluble cytoplasmic markers (Johnson & Whittaker, 1963; Ryall, 1964). Johnson & Whittaker (1963) found a somewhat sharper localization of particulate lactate dehydrogenase in fraction B than reported here; this is due to the fact that in their experiments fraction P_2 was washed with sucrose to remove the fraction of soluble cytoplasm, which was entrapped within myelin fragments. Unlike the synaptosome cytoplasm, this is readily washed out by sucrose.

Table 4 shows the concentrations of amino acids, lactate dehydrogenase and potassium relative to the concentration of total nitrogen in the fractions. These values were obtained by dividing the distribution of the various components expressed as a percentage of the total recovered in the various fractions by the distribution of nitrogen expressed in similar units. The values for the amino acids were obtained from the means of the individual distribution values, a procedure justified by the closeness of these values to each other. The only fraction showing an enrichment of these components relative to the original homogenate (relative concentration, 1·00) is the soluble supernatant (S_3) fraction.

The subcellular distributions of the materials in peaks 1–3 have no great significance, since each peak comprises a complex mixture of substances. However, the material in peak 3 had a subcellular distribution closely similar to those of the non-phosphorus-containing amino compounds. The materials of peak 2 appeared to show a preferential localization in fraction B. However, the size of this peak in the chromatograms of the initial homogenate and the various fractions was low and variable and the 'recovery' was considerably in excess of 100%, suggesting autolytic breakdown. Further work will be necessary to determine whether there

is a component or components present in peak 2 that has a definite localization in fraction *B*.

Redistribution of amino acids during fractionation. Although there appeared to be no appreciable net synthesis or destruction of most of the amino acids during fractionation, as shown by recoveries at the end of the experiments close to 100%, the possibility was considered that redistribution of the amino acids could have taken place during fractionation either by leakage from particulate material into the supernatant or by adsorption from the supernatant on to particulate material. Comparisons were therefore made between the ratio of the total amounts recovered in the high-speed supernatant and the particulate fractions when complete fractionation was carried out and that obtained when the homogenate was centrifuged directly at 100000*g* for 60 min. to give a high-speed supernatant (*S*) and a total particulate fraction (*P*). Table 5 shows that some leakage does take place during fractionation or even during standing at 0°: the *S*/*P* ratio increased from 1·94 when the homogenate was submitted to high-speed centrifuging immediately after preparation to 2·45 after standing for 2–6 hr. as compared with a ratio 2·70 as a result of full fractionation. These increases are smaller than those observed with hydroxytryptamine (Michaelson & Whittaker, 1963).

Effect of homogenizing in saline. Elliott & van Gelder (1960) reported that 'factor I' (γ-aminobutyrate) remained 70% bound in saline homogenates but only 30% bound in sucrose. We therefore determined one series of *S*/*P* ratios by using a homogenate made in 0·9% sodium chloride. The results are presented in Table 5. Except for the amides, the amino acid content was somewhat lower than in the experiments presented in Table 2, but the general pattern of distribution was similar; 63% or more of the amino acids was recovered in the high-speed supernatant. The proportion in the supernatant was, however, usually somewhat lower in saline than in sucrose.

Distribution of amino acids in subfractions from disrupted synaptosomes

The results so far presented show that the free amino acids of brain tissue have a subcellular distribution similar to that of soluble cytoplasmic markers such as lactate dehydrogenase and potassium. Their presence in the synaptosome and other particulate fractions could be accounted for by the presence in these fractions of entrapped cytoplasm. It was therefore decided to study the distribution of the amino acids in density-gradient fractions of disrupted synaptosomes, to see whether the amino acids again had a distribution similar to the soluble cytoplasmic markers. Particular attention was paid to the synaptic-vesicle fraction because of the presence of bound acetylcholine in this fraction and the possible role of the synaptic vesicles in the binding of transmitter substances generally.

Two series of experiments were carried out, with synaptosomes prepared from whole brain and

Table 5. *Distribution of amino acids between particulate and high-speed supernatant fractions*

The supernatant/particulate ratio for the first entry is the ratio $S_3/(P_1+A+B+C+P_3)$ calculated from the results given in Table 2, and for the remaining entries it is *S*/*P*. For nomenclature of fractions see the text.

Amino acids	Time from homogenizing to final sampling (hr.)	Concn. in homogenate (μmoles or μg.atoms)	Amino acids recovered in fractions (% of homogenate)	Supernatant/particulate concentration ratio
Full fractionation				
Total non-P-containing amino acids	5	15·94	96	2·70
High-speed centrifuging only				
Total carboxylic amino N	1	16·22	111	1·94
	3		115	2·45
	7		102	2·45
High-speed centrifuging only, saline homogenate				
Non-P-containing amino acids:				
Glutamate	1	5·66	104	2·50
Amides		3·52	69	2·51
Aspartate		2·23	88	2·33
γ-Aminobutyrate		1·47	104	1·78
Serine		0·74	83	2·07
Glycine		0·69	100	1·96
Alanine		0·55	116	2·86
Threonine		0·09	120	2·09
Total non-P-containing amino acids		14·95	94	2·33

Table 6. *Distribution of amino nitrogen and marker substances in subfractions of disrupted synaptosomes*

For nomenclature of fractions see the text.

Component	No. of experiments	Units	Parent fraction	Amount recovered (units/g. of original tissue)	Amount recovered (% of parent fraction)	Distribution (% of recovered amount) *O*	*D*	*E*	*F*	*G*	*H*	*I*	*J*	D_s	D_p
Whole brain															
Amino N	1	μg.atoms	W_s	3·34	102	55	17	7	6	6	7	2	—	—	—
Lactate dehydrogenase*	3	μmoles of $NADH_2$ disappearing/min.	W_s	5·32	71	74	16	3	1	0	0	3	—	—	—
Potassium*	1	μg.atoms	W_s	6·50	100	75	14	4	5	2	0	0	—	—	—
Acetylcholine*	4	mμmoles	W_s	1·66	81	2	30	8	5	11	35	9	—	—	—
Total nitrogen*	11	mg.atoms	W_s	0·17	84	28	12	8	12	12	15	13	—	—	—
Cortex															
Amino N	2	μg.atoms	P_2	4·00	84	52	21	—	—	—	—	—	27	—	—
	2	μg.atoms	D	0·72	89	—	—	—	—	—	—	—	—	73	27
Potassium	1	μg.atoms	P_2	8·67	87	55	19	—	—	—	—	—	26	—	—
Acetylcholine	1	mμmoles	P_2	5·56	84	8	49	—	—	—	—	—	43	—	—
Total N	3	mg.atoms	P_2	0·18	82	20	10	—	—	—	—	—	70	—	—

* Results of Whittaker *et al.* (1964) included for comparison.

cortex respectively. The results are summarized in Table 6. Most of the amino nitrogen was recovered in each series in fraction *O* containing the soluble cytoplasmic component of the synaptosomes. In the whole-brain series, the distribution is similar to that of lactate dehydrogenase and potassium, except that relatively more is present in the fractions containing membrane fragments (*E*–*G*) and incompletely disrupted synaptosomes (*H*). The amount of all these substances present in fraction *D* can be accounted for by diffusion from fraction *O*. The distribution contrasts with that of bound acetylcholine (l. 4), which shows a bimodal distribution with peaks in the fractions containing synaptic vesicles (*D*) and incompletely disrupted synaptosomes (*H*). A comparison with the total nitrogen distribution shows that the maximum concentration of amino acids with respect to other nitrogenous constituents of the fractions was also in fraction *O*.

In the series with disrupted cortical synaptosomes, a similar trend was evident, the distribution in the gradient being similar to potassium and the richest fraction being the soluble cytoplasmic fraction *O*. The contrast with bound acetylcholine was even more marked, almost half of the latter being recovered in the synaptic-vesicle fraction.

However, the possibility still remained that the synaptic vesicles might have concentrated particular amino acids from the mixture of free amino acids present in the synaptosome fraction whose presence there would have been masked by the larger amount of free amino acids recovered in fraction *O*. Consequently in five experiments the *D* fraction from brain cortex was diluted with an equal volume of water and centrifuged at 100000*g* for 60 min. to sediment the synaptic vesicles. The amino acid distribution was then determined in the precipitate and supernatant (fractions D_p and D_s). In previous work with whole brain (Whittaker *et al.* 1964) 80% of the recovered acetylcholine had sedimented into fraction D_p. In three out of five of these experiments an interfering substance was released into fraction

Table 7. *Composition of free amino acid pool in subfractions of synaptosomes from guinea-pig cortex*

For nomenclature of fractions see the text.

	Percentage composition of amino acid pool P_2	*O*	D_s
Glutamate	37	35	31
Amides	24	13	10
Aspartate	15	5	10
γ-Aminobutyrate	7	20	5
Serine	6	13	11
Glycine	5	10	21
Alanine	4	4	7
Threonine	2	<1	5
Total amino N (μmoles/g.)	3·29	1·23	0·28
As % of total amino compounds present in fraction	71	59	53

D_s during this procedure that did not appear as an amino acid peak on subsequent chromatography. Consequently the total amino nitrogen of this fraction could not be determined by the ninhydrin method and was instead obtained by summing the amino acid peaks. The distribution studies (Table 6, l. 7, columns 15 and 16) indicated that only 27% of the total amino acid originally in the synaptic-vesicle fraction sedimented with the synaptic vesicles. This represents less than 0·5% of the amino acid nitrogen of the original homogenate.

Amino acid analyses of cortical fractions P_2, O and D_s (Table 7) showed that in all three fractions the same eight amino acids accounted for 53–71% of the total amino compounds with glutamate as the most abundant. The low concentration of ninhydrin-positive material in fraction D_p did not permit an analysis of this fraction.

DISCUSSION

Seven amino acids together account for over 80% of the free amino compounds of sucrose homogenates of guinea-pig brain. These are, in decreasing order of abundance: glutamate, aspartate, γ-aminobutyrate, glycine, serine, alanine and threonine. In addition, there are appreciable quantities of amide (presumably glutamine).

Of these compounds, glutamate and aspartate can stimulate nerve cells to discharge electrically when applied iontophoretically by means of microelectrodes, and γ-aminobutyrate, alanine and glycine, again in decreasing order of effectiveness, depress central neurones when similarly applied (Curtis & Watkins, 1960; Krnjević, 1965). The uniquely high concentrations of glutamate and γ-aminobutyrate, respectively the most effective excitant and depressant compounds of this class found in brain tissue, have led to speculations that these compounds may be the chemical transmitter substances at the many excitatory and inhibitory endings at which no other transmitter has so far been implicated.

The subcellular fractionation studies do not, however, demonstrate, as they did for acetylcholine, a selective localization of these, or, indeed, of any of the other amino acids in the fraction in which the isolated nerve endings are concentrated; 69–82% was recovered in the high-speed supernatant fraction and the distribution in the other fractions closely parallels that of the soluble cytoplasmic markers lactate dehydrogenase and potassium. This finding is in agreement with the results of Ryall (1964) for glutamate and γ-aminobutyrate in rat brain and of Weinstein *et al.* (1963) for γ-aminobutyrate in mouse brain. A reasonable inference is that the amino acid content of the particulate fractions merely reflects the occlusion of cytoplasm within organized cell fragments (mainly nerve endings) in these fractions.

The results of hypo-osmotic disruption of synaptosomes (Table 6) confirm these conclusions; over half (52–55%) of the amino nitrogen of the preparations was recovered in the fraction (O) corresponding to soluble cytoplasm, compared with 55–75% of the potassium. The next richest fraction was that containing the synaptic vesicles (D), which have been shown to contain acetylcholine; but when the vesicles were sedimented along with their contained acetylcholine most (73%) of the amino compounds remained in the supernatant. No evidence was obtained for a selective localization of any particular amino acid in any of these subfractions; pharmacologically active and inactive amino acids were distributed in an essentially similar way.

These findings, together with the fact that no amino acid so far tested has duplicated exactly the post-synaptic electrical effects of synaptic transmission (Curtis, 1962, 1963), have been regarded as evidence against a specific transmitter role for these substances in the mammalian central nervous system (Crawford & Curtis, 1964). Nevertheless, a note of caution should be sounded with regard to the interpretation of subcellular distribution studies. Although a relatively small proportion of the total tissue glutamate and γ-aminobutyrate is recovered in the synaptosome and synaptic-vesicle fractions, Krnjević & Whittaker (1965) directly demonstrated by means of electroendosmosis from micropipettes that there is sufficient of both these substances in extracts of these fractions to produce effects on single cortical neurones of the guinea pig. From synaptosome (F. Clementi, M. N. Sheridan & V. P. Whittaker, unpublished work) and synaptic-vesicle (Whittaker & Sheridan, 1965) counts they were able to estimate that excitant effects were being obtained with rates of glutamate release corresponding to the content of about 700 synaptosomes/sec. and depressant effects with rates of γ-aminobutyrate release corresponding to that of about 350 synaptosomes/sec. Synaptic activation at these rates was considered to be well within the possible physiological limit.

There is, in fact, no reason to assume that the mechanisms of storage and release of all central transmitters must be the same as those of acetylcholine. The storage mechanism might, for example, involve continuous active uptake; under the conditions of subcellular fractionation in an essentially ion-free medium at 0°, outward diffusion from storage sites might occur, leading to a distribution of transmitter indistinguishable from that of a soluble cytoplasmic constituent. Sano & Roberts (1963) have obtained evidence for a Na^+-dependent uptake of labelled γ-aminobutyrate in

mouse-brain particles; a more detailed analysis (Varon, Weinstein & Roberts, 1964) suggested that endogenous γ-aminobutyrate is concomitantly released into the incubation medium. It is thus noteworthy that the γ-aminobutyrate-forming enzyme glutamate decarboxylase, which, as a high-molecular-weight substance, may be less liable to redistribution during fractionation, has been reported to be localized in a dense synaptosome fraction (Weinstein *et al.* 1963; Salganicoff & De Robertis, 1963; van den Berg, 1964).

Any transmitter that is not highly concentrated at nerve endings will show a distribution approximating to that of a soluble cytoplasmic marker if the neurones containing the transmitter are represented in the sample as a whole and not simply by their nerve endings (for discussion see Laverty, Michaelson, Sharman & Whittaker, 1963; Krnjević & Whittaker, 1965). Inhibitory endings, particularly, are likely to originate according to recent views (Eccles, 1964) from cells (e.g. basket or stellate cells) with short axons making axosomatic contacts with neighbouring cells; thus the inhibitory neurones as a whole will be included in the preparation. It is also not clear to what extent axosomatic as contrasted with axodendritic endings are pinched off intact during homogenization. In view of all these considerations it is unlikely that the evidence from subcellular distribution is critical enough at the present time to provide an adequate basis for rejecting glutamate and γ-aminobutyrate as transmitters.

In the course of the present work, the presence in brain homogenates of appreciable amounts of a peptide that undergoes spontaneous hydrolysis to glutamate and of at least 18 unidentified ninhydrin-positive compounds was noted. Apart from the peptide the subcellular distribution of none of these substances has been followed up, nor is it known if any of them have effects on central neurones.

This work was supported by Grant no. NB-03928 from the National Institute of Neurological Diseases and Blindness of the National Institutes of Health, U.S. Public Health Service. We express our appreciation to Miss J. Willmott, Mr G. H. C. Dowe and Mr. P. Wright for their expert technical assistance, to Miss N. Hemington for paper chromatography and to Dr R. J. A. Hosie (née Kirkland) for performing some of the lactate-dehydrogenase assays. The amino acid analyser was constructed in the Institute workshops by Mr D. V. Barker, Mr C. Ryder and Mr J. S. Chilvers.

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The Biological Sulphation of L-Tyrosyl Peptides

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(*Received* 18 *June* 1965)

1. A rat-liver supernatant preparation can achieve the biological *O*-sulphation of L-tyrosylglycine and L-tyrosyl-L-alanine at pH 7·0. 2. The optimum concentrations of L-tyrosylglycine and L-tyrosyl-L-alanine in this system are 50mM and 60mM respectively. 3. L-Tyrosylglycine yields two sulphated products, whereas L-tyrosyl-L-alanine yields three sulphated products, when used as acceptor for sulphate in the rat-liver system. 4. With both substrates, one of the sulphated products has been identified as the *O*-sulphate ester of the corresponding parent peptide.

L-Tyrosine *O*-sulphate has been identified as a component of mammalian urines (see John, Rose, Wusteman & Dodgson, 1965), of mammalian fibrinogens (see Doolittle & Blombäck, 1964) and of gastrin II isolated from hog antral mucosa (Gregory, Hardy, Jones, Kenner & Sheppard, 1964). Attempts made to achieve the biological sulphation of free L-tyrosine have all failed (Dodgson, Rose & Tudball, 1959; Nose & Lipmann, 1958; Segal & Mologne, 1959; Suzuki & Strominger, 1960; Vestermark & Boström, 1959). However, it has been established that the methyl and ethyl esters of L-tyrosine will act as sulphate acceptors in a rat-liver sulphating system (Segal & Mologne, 1959; Jones & Dodgson, 1965). Thus it has been suggested that naturally occurring carboxyl-substituted derivatives of L-tyrosine may act as sulphate acceptors *in vivo* and that small peptide fragments with L-tyrosine in the *N*-terminal position may well act in this capacity. Segal & Mologne (1959) measured the transfer of sulphate from *p*-nitrophenyl sulphate via adenosine 3′,5′-diphosphate to L-tyrosylglycine by using a partially purified phenol-sulphotransferase preparation from rat liver. They detected weak transferase activity at pH 9·3 but none at pH 7·8. The present paper demonstrates the sulphation of L-tyrosylglycine and L-tyrosyl-L-alanine in a rat-liver supernatant preparation at pH 7·0, with inorganic sulphate and ATP as sources of sulphate and energy respectively. Preliminary accounts of this work have already been given (Jones, Scotland & Dodgson, 1965*a*,*b*).

MATERIALS AND METHODS

Sulphate esters. The potassium salt of L-tyrosine *O*[^{35}S]-sulphate was prepared according to the method described by Dodgson, Powell, Rose & Tudball (1961). L-Tyrosylglycine (500mg.; Yeda Research and Development Co. Ltd., Rehovoth, Israel) was sulphated by a method similar to that described by Dodgson *et al.* (1959) for the sulphation of glycyl-L-tyrosine. However, the total reaction time was reduced from 40min. to 15min. and the final product was precipitated by dissolving it in 100μl. of water and adding the solution to 5ml. of ethanol. After 30min. at 4° the crystals of monopotassium L-tyrosylglycine *O*-sulphate were separated, washed with 5ml. of ethanol followed by 5ml. of ether and dried *in vacuo* over P_2O_5 at 100° for 12hr. The yield was 20mg. (Found: ester SO_4^{2-}, 25%; K^+, 10·3%. Monopotassium L-tyrosylglycine *O*-sulphate, $C_{11}H_{13}KN_2O_7S$, requires ester SO_4^{2-}, 27%; K^+, 10·9%).

L-Tyrosyl-L-alanine (500mg.; Yeda Research and Development Co. Ltd.) was sulphated by the procedure described for L-tyrosylglycine. The yield was 40mg. (Found: ester SO_4^{2-}, 25·8%; K^+, 11·0%. Monopotassium L-tyrosyl-L-alanine *O*-sulphate, $C_{12}H_{15}KN_2O_7S$, requires ester SO_4^{2-}, 26·0%; K^+, 10·5%).

The homogeneity of the preparations of the sulphate esters was checked by descending chromatography with Whatman no. 1 paper, the solvent (solvent system *A*) being butan-1-ol–acetic acid–water (50:12:25, by vol.). After 16hr. the chromatograms were dried and developed by spraying with a 0·25% (w/v) solution of ninhydrin in acetone and drying in air at 100°. The R_F values of the parent compounds and their sulphate esters were: L-tyrosine, 0·45; L-tyrosine *O*-sulphate, 0·20; L-tyrosylglycine, 0·42; L-tyrosylglycine *O*-sulphate, 0·24; L-tyrosyl-L-alanine, 0·48; L-tyrosyl-L-alanine *O*-sulphate, 0·33. Hydrolysis of each sulphate ester in N-HCl at 100° for 4min. yielded inorganic sulphate and the corresponding amino acid or peptide. The ultraviolet-absorption spectra of the parent peptides and their sulphate esters were identical with those of L-tyrosine and L-tyrosine *O*-sulphate respectively. This is in accord with the conclusion that the compounds described above are true *O*-sulphate esters (see Dodgson *et al.* 1959).

Preparation of enzymes. Rat-liver whole supernatant was prepared by the method described by Jones & Dodgson (1965) and stored in 1·0ml. portions at −10°.

Incubation mixture. The standard incubation mixture contained 100μl. of enzyme preparation, 4μmoles of ATP (disodium salt; Sigma Chemical Co., St Louis, Mo., U.S.A.), 2μmoles of $MgCl_2$, 1μmole of cysteine hydrochloride, 10μc of $Na_2{}^{35}SO_4$ (carrier-free: code SJS1, The Radiochemical Centre, Amersham, Bucks.), 5μmoles of KH_2PO_4 and sulphate acceptors according to the plan of the experiment (the acceptor compounds were either dissolved in the solution of KH_2PO_4 or, for amounts greater than 2μmoles, they were added as solid to the incubation mixture). The pH of all components was adjusted to 7·0 with 2N-NaOH and the final mixture (volume 170μl.) was incubated for 1hr. at 38°. After incubation the reaction tubes were immersed in boiling water for 2min. and the precipitated protein was removed by centrifuging.

Paper chromatography and electrophoresis. Samples (6μl.) of deproteinized incubation mixtures were applied to Whatman no. 1 paper and subjected to descending chromatography for 16hr. at room temperature with solvent system *A* or isobutyric acid–0·3N-NH_3 (5:3, v/v) (solvent system *B*). Samples (6μl.) were also subjected to electrophoresis on Whatman no. 1 paper for 2hr. in 0·1M-sodium acetate–acetic acid buffer, pH4·5, with a potential gradient of 11v/cm. Under these conditions L-tyrosine *O*-sulphate moved 3·2cm., L-tyrosylglycine *O*-sulphate moved 2·3cm. and L-tyrosyl-L-alanine *O*-sulphate moved 2·0cm.

Detection and estimation of radioactivity. Radioactive spots were located on dried chromatograms and electrophoresis strips by exposure to Ilford Industrial B X-ray film for 3 days or by scanning with the C–100 Actigraph automatic chromatogram scanner (Nuclear–Chicago Corp., Des Plaines, Ill., U.S.A.). The relative amount of radioactivity associated with each spot was estimated from the record of the scanner as described by Jones & Dodgson (1965).

EXPERIMENTAL AND RESULTS

In the absence of added acceptor the enzyme preparation synthesized ^{35}S-labelled 3′-phosphoadenylyl sulphate which was characterized by chromatography in solvent systems *A* and *B* and by paper electrophoresis.

The enzyme preparation was able to use *p*-nitrophenol, L-tyrosine methyl ester or L-tyrosine ethyl ester as sulphate acceptor, but could not use L-tyrosine or glycyl L-tyrosine.

Enzymic synthesis of L-*tyrosylglycine* O[^{35}S]-*sulphate by the rat-liver sulphating system.* L-Tyrosylglycine was incubated in the standard reaction mixture for 1hr. at 38°. The amounts of the peptide were varied so as to give final concentrations in the reaction mixture varying from 0 to 60mM. At the end of the incubation period, samples (6μl.) of deproteinized mixtures were examined by paper chromatography in solvent system *A* and by paper electrophoresis.

Radioautography of dried chromatograms and electrophoresis strips indicated that incubation mixtures, with L-tyrosylglycine as sulphate acceptor, contained two new radioactive compounds in addition to ^{35}S-labelled inorganic sulphate and 3′-phosphoadenylyl sulphate. The chromatographic mobility of the major compound (metabolite I; R_F 0·24 in solvent system *A*) was identical with that of authentic L-tyrosylglycine *O*-sulphate. Similar agreement was obtained between the electrophoretic mobility of this metabolite and that of L-tyrosylglycine *O*-sulphate. The second radioactive metabolite detected on chromatograms (metabolite II) had R_F 0·28 in solvent system *A*. The second radioactive compound detected on electrophoretograms (assumed to be metabolite II) possessed no net charge when examined by paper electrophoresis at pH4·5. L-Tyrosine *O*[^{35}S]-sulphate was not detected on chromatograms or electrophoretograms. The amount of radioactivity associated with both metabolites was assayed as described above by scanning the paper chromatograms. Owing to the close proximity of the spots, the combined area under the peaks on the recording chart was taken as a measure of the sulphation of L-tyrosylglycine. The results (Fig. 1) show that the transfer of [^{35}S]sulphate to L-tyrosylglycine proceeds maximally when the concentration of the acceptor is approx. 50mM. A visual appraisal of radioautograms indicated that the relative amounts of both metabolites increased in parallel with increasing concentration of L-tyrosylglycine. Finally, chromatograms (developed with 0·25% ninhydrin in acetone) exhibited spots corresponding in mobilities to free L-tyrosine (R_F 0·45) and glycine (R_F 0·23).

Metabolite I was then separated from 0·5ml. of pooled deproteinized reaction mixtures by paper chromatography (solvent system *A*) and the appropriate areas were eluted with water. To the

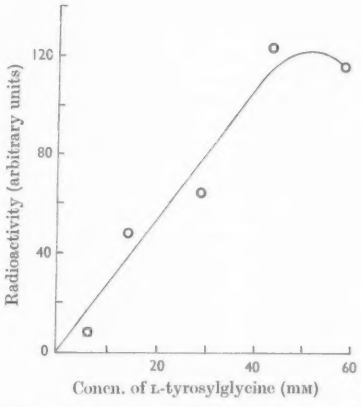

Fig. 1. Substrate concentration–activity curve for the sulphation of L-tyrosylglycine by rat-liver whole supernatant. Details are given in the text.

combined eluates (approx. 10ml.) was added 15mg. of authentic carrier L-tyrosylglycine *O*-sulphate and the whole was applied to a column (10cm. × 1cm.) of Dowex 50 resin (H^+ form; 20–50 mesh). This latter procedure was necessary to remove glycine, which travels with metabolite I on paper chromatography in solvent system *A*. The column of resin was then washed with 20ml. of water, and the eluate and washings were neutralized with N-potassium hydroxide, concentrated *in vacuo* at 40° to approx. 0·5ml. and added to 5ml. of ethanol. The white solid that was precipitated was collected by centrifuging, washed with 5ml. of ethanol followed by 5ml. of ether and finally dried *in vacuo* over phosphorus pentoxide at room temperature (the yield was approx. 10mg.). The product was then examined by descending paper chromatography in solvent systems *A* and *B*, by two-way paper chromatography with phenol–water (4:1, w/v) as first solvent and solvent system *A* as second solvent and by paper electrophoresis under the conditions described above. All methods showed a single spot, which was radioactive, gave a positive ninhydrin reaction, absorbed ultraviolet light and moved as expected for L-tyrosylglycine *O*-sulphate.

Finally, the product was dissolved in saturated sodium hydrogen carbonate solution (2mg./0·5ml.) and added to 0·5ml. of ethanol containing 10mg. of 1-fluoro-2,4-dinitrobenzene. The mixture was shaken at room temperature for 2hr. and then evaporated to dryness *in vacuo*. The residue was dissolved in 1ml. of water and examined by paper chromatography in solvent system *A*. Chromatograms showed the presence of a single DNP derivative, which was also radioactive.

Enzymic synthesis of L-*tyrosyl*-L-*alanine* O[^{35}S]-*sulphate by the rat-liver sulphating system.* L-Tyrosyl-L-alanine was incubated in the standard reaction mixture for 1hr. at 38°. The final concentration of the peptide in the reaction mixture varied from 0 to 120mM. At the end of the incubation period the deproteinized mixtures were examined by paper chromatography and electrophoresis followed by radioautography and scanning in the usual way. Two new radioactive metabolites were detected in incubation mixtures when the concentration of L-tyrosyl-L-alanine was between 10mM and 30mM, whereas a third metabolite was detected when the concentration of the peptide was 60mM and 120mM. The chromatographic mobility of one of these metabolites (metabolite 1; R_F 0·33 in solvent system *A*) was identical with that of authentic L-tyrosyl-L-alanine *O*-sulphate. Similar agreement was obtained between the electrophoretic mobilities of these two compounds. No free L-tyrosine O[^{35}S]-sulphate was detected on chromatograms or electrophoretograms, but the behaviour of the other two radioactive metabolites on paper

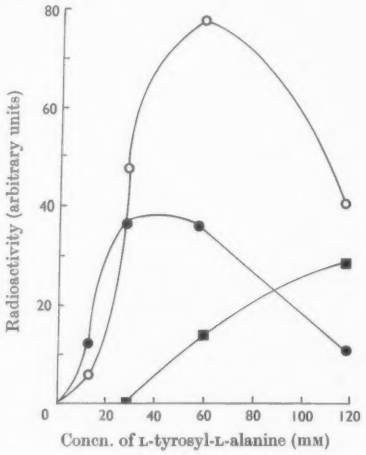

Fig. 2. Substrate concentration–activity curves for the sulphation of L-tyrosyl-L-alanine by rat-liver whole supernatant. ○, Production of L-tyrosyl-L-alanine *O*[^{35}S]-sulphate (metabolite 1); ●, production of metabolite 2; ■, production of metabolite 3. Details are given in the text.

electrophoresis suggests that one of them (metabolite 2; R_F 0·22 in solvent system *A*) is less acidic and the other (metabolite 3; R_F 0·43 in solvent system *A*) is more acidic than L-tyrosyl-L-alanine *O*-sulphate. The relative amounts of each metabolite in incubation mixtures were estimated by scanning chromatograms in the usual way. The results (Fig. 2) show that the optimum concentration of L-tyrosyl-L-alanine for this sulphating system is approx. 60mM. Chromatograms (developed with 0·25% ninhydrin in acetone) showed the presence of L-tyrosine (R_F 0·45 in solvent system *A*) and L-alanine (R_F 0·31 in solvent system *A*).

Metabolite 1 was separated from 100μl. of pooled deproteinized incubation mixtures by paper chromatography followed by ion-exchange chromatography in the presence of 25mg. of authentic carrier L-tyrosyl-L-alanine *O*-sulphate, the whole procedure being carried out as described above for the isolation of L-tyrosylglycine *O*-sulphate. The final white solid (approx. 20mg.) was precipitated twice from 100μl. of water by the addition of 5ml. of ethanol. After each precipitation a small sample (approx. 1mg.) was retained for estimation of its specific activity as described by Dodgson *et al.* (1961), and for examination by paper electrophoresis. Both materials had identical specific activities (100 counts/min./mg.) and, when examined by electrophoresis, gave one spot, which was radioactive, gave a positive ninhydrin reaction and absorbed ultraviolet light.

DISCUSSION

The present work establishes that L-tyrosylglycine and L-tyrosyl-L-alanine can act as acceptors for sulphate in the 3′-phosphoadenylyl sulphate–phenol-sulphotransferase system (EC 2.8.2.1) of rat-liver at pH 7·0. In contrast, glycyl-L-tyrosine could not act as sulphate acceptor in this system.

The optimum concentration of acceptors for the rat-liver sulphating system was approx. 50mM and approx. 60mM for L-tyrosylglycine and L-tyrosyl-L-alanine respectively. However, these values undoubtedly reflect, to some extent, the presence of enzymes in the system that hydrolyse both peptides to their constituent amino acids. Incubation of L-tyrosylglycine or L-tyrosyl-L-alanine with the rat-liver sulphating system leads to the appearance of the corresponding ^{35}S-labelled *O*-sulphate ester among the products formed. These findings support the view that small peptide fragments with L-tyrosine in the *N*-terminal position may act as sulphate acceptors *in vivo*. Further, it is possible that these sulphated peptides may be hydrolysed *in vivo* to yield free L-tyrosine *O*-sulphate and hence account, in part, for the occurrence of this sulphated amino acid in mammalian urines. In this connexion it has been shown (Jones, Dodgson, Powell & Rose, 1963) that rabbit fibrinopeptide B, which contains one residue of L-tyrosine *O*-sulphate, is metabolized by the rabbit to yield free L-tyrosine *O*-sulphate, which can then be detected in the urine.

It is not yet clear whether the other radioactive compounds, detected in incubation mixtures when either L-tyrosylglycine or L-tyrosyl-L-alanine was used as sulphate acceptor, represent breakdown products of the sulphated peptides or sulphated products of degraded fragments of the original peptides.

This work was supported by the Medical Research Council and by the U.S. Public Health Service Research Grant no. AM01982-07 from the Arthritis and Metabolic Diseases Division. S. M. S. is grateful to the Medical Research Council for a research assistantship. We thank the Royal Society for a gift of the Optica CF4R recording spectrophotometer.

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The Chemistry of Xanthine Oxidase

REACTION WITH IODOACETAMIDE

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(*Received* 26 *April* 1965)

1. The reaction of milk xanthine oxidase with iodoacetamide has been studied with the silver–silver iodide electrode. 2. The reaction proceeds considerably faster in the presence of xanthine than in its absence. Anaerobically, with excess of xanthine, the reaction takes place as a rapid phase in which the enzyme is inactivated and in which approx. 1 thiol group/mol. of enzyme reacts and as a slower phase in which about 12 groups/mol. react. 3. The rapid reaction appears to be first-order with respect to xanthine oxidase and iodoacetamide and independent of the xanthine concentration with more than about 3 mol. of xanthine/mol. of enzyme. 4. The velocity constant of the rapid phase is 0·26 min.$^{-1}$ at 25° and pH 7·0, with 1 mM-iodoacetamide and 17 μM-xanthine oxidase. The velocity constant for the slower phase is about one-hundredth of this value. 5. The velocities of both phases increase with increasing pH in the range 5·0–9·6. 6. Xanthine may be replaced by salicylaldehyde without affecting the rate of loss of enzymic activity. With sodium dithionite as reducing agent, the reaction is slightly faster. 7. The possible function of thiol groups in the reaction mechanism of the enzyme is discussed.

The importance of thiol groups in the reaction mechanism of milk xanthine oxidase is implied by the work of Mackworth (1948), Harris & Hellerman (1956), Wills (1956), Peters & Sanadi (1961) and Gilbert (1963). Bergel & Bray (1959) found that, of at least 35 groups in the enzyme that could react with sodium *p*-chloromercuribenzoate, 9 groups reacted relatively rapidly (half-reaction time about 2 hr. at 24°) but without affecting the enzymic activity. In contrast with this, Fridovich & Handler (1958) found that in the presence of the substrate the enzyme was rapidly inactivated by the mercurial. However, they did not study the stoichiometry of the reaction. Their work was confirmed by Brumby, Miller & Massey (1965) with the liver enzyme. These workers also titrated this enzyme amperometically with phenylmercuric acetate and reported that, when the enzyme was reduced with hypoxanthine, the titre increased by about 4 thiol groups/mol. of enzyme flavine. Gilbert (1962) found that the milk enzyme was stable in the presence of iodoacetamide, but that on adding xanthine rapid inactivation took place.

The present investigation describes quantitative studies, with the silver–silver iodide electrode (Watts, Rabin & Crook, 1961), on the reaction with iodoacetamide of those reactive groupings in the milk enzyme that appear on reduction. A single thiol group in the molecule is implicated. The possible role of such a group in the catalytic mechanism and the group's relationship to other components of the enzyme are discussed.

MATERIALS

Chemicals. Iodoacetamide (from L. Light and Co. Ltd., Colnbrook, Bucks.) was recrystallized five times from aq. 50% (v/v) ethanol, as described by Watts *et al.* (1961). Phenylxanthine was a gift from Professor F. Bergmann. All other reagents were of A.R. grade where obtainable.

Buffers. Pyrophosphate buffer was prepared from the di- and tetra-sodium salts; acetate buffer was prepared from acetic acid and NaOH (Datta & Grzybowski, 1961); tris-maleate and carbonate buffers were prepared as described by Gomori (1955).

Xanthine oxidase. The enzyme was prepared from milk as described by Gilbert & Bergel (1964) and by Palmer, Bray & Beinert (1964), and high-molecular-weight impurities were removed by gel filtration on a column of Sephadex G-200 (cf. Andrews, Bray, Edwards & Shooter, 1964). The samples used generally had activity/E_{450} values approx. 100 (cf. Bray, Pettersson & Ehrenberg, 1961), but in one case (Fig. 7) the value was only 50. This sample had a molybdenum content, determined by a modification (L. I. Hart, unpublished work) of the dithiol method, of 0·9 atom of Mo/mol. of xanthine oxidase, whereas another sample (Fig. 4), with activity/E_{450} 100, had 1·3 atoms of Mo/mol. of xanthine oxidase. Xanthine oxidase concen-

trations were calculated from E_{450} values by taking ϵ to be 70000. For anaerobic experiments, the enzyme was concentrated by vacuum-dialysis against a suitable buffer containing EDTA (as shown in the legends to the Figures) and the solution (approx. 0·25mM) was made anaerobic by evacuating and flushing the tube in which it was contained several times with argon purified with vanadous sulphate (Meites & Meites, 1948). The enzyme was then drawn into a syringe with a Teflon plunger (Hamilton Co., Whittier, Calif., U.S.A.) previously flushed with argon, and was dispensed from this syringe into the reaction vessel (see below).

METHODS

Reaction with iodoacetamide. For the aerobic experiments the reaction was carried out as described by Watts *et al.* (1961), with a silver–silver iodide electrode assembly to follow the production of I^- ions, with a total volume of 4·0–4·7ml. at 25°. For the anaerobic experiments a special cell was constructed (Fig. 1) and a reaction volume of 2·3ml. was employed. Reagents, as described in the legends to the Figures, were pipetted into the cell and deoxygenated by bubbling purified argon through the mixture for 15min. The bubbler (*B* in Fig. 1) was then withdrawn and argon was blown rapidly over the surface of the reaction mixture via G_1 (Fig. 1), to exclude oxygen. (Argon was preferred to nitrogen for this purpose owing to its greater density.) The reaction was initiated by momentarily stopping the stirrer, removing the stopper (*S* in Fig. 1) and adding either deoxygenated xanthine oxidase solution from a syringe (see the Materials section) or deoxygenated xanthine from a pipette previously flushed with argon. Samples of the reaction mixture for enzyme assay were withdrawn with a pipette in a similar manner and delivered rapidly into an excess of ice-cold cysteine solution (2mM in 50mM-pyrophosphate buffer, pH 8·2) to quench the alkylation reaction. In some experiments the iodide production curves showed signs of a short induction period. Since this was probably due to slow mixing of the dense xanthine oxidase solution into the reaction medium, a 0·5min. correction was applied to the times where appropriate.

The anaerobic technique was similar when iodide production was not followed and only activity measurements were made, but the total volume was decreased to 0·5ml. Sodium dithionite solution was prepared and standardized as described by Bray *et al.* (1961).

Assay of xanthine oxidase. Activity was determined spectrophotometrically, with xanthine as substrate, as described by Bergel & Bray (1959), on portions of the reaction mixture withdrawn and diluted as described above.

RESULTS

Aerobic experiments

It was found (Fig. 2) that the enzyme liberated I^- ions from iodoacetamide more rapidly when xanthine was added to the reaction mixture than it did in the absence of a substrate. However, after a short period, i.e. at about the time when it would be expected that all the xanthine had been oxidized by the enzyme, the rate of iodide production fell

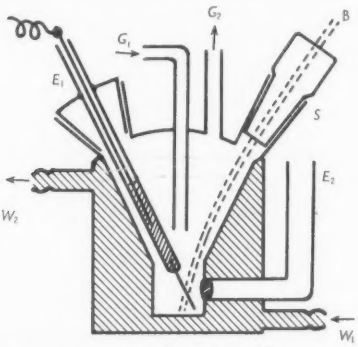

Fig. 1. Apparatus used for following the anaerobic reaction with iodoacetamide. The constant-temperature water jacket (inlet: W_1; outlet: W_2) surrounds a magnetically stirred central reaction chamber, with 1·4cm. diam. and 2·5ml. capacity. The iodide electrode, E_1, is inserted through a standard B14 joint in the top of the vessel and the satd. KNO_3 bridge, E_2, into which is inserted the calomel reference electrode, is fused through the water jacket into the side of the reaction chamber. Mixing is prevented by a sintered plug. Argon is blown over the surface of the reaction mixture via G_1 or is bubbled through it via a removable polythene bubbling tube, *B*, which passes through a B10 joint, *S*. Gas escapes through G_2. The B10 stopper is also used for making additions to, or removing samples from, the reaction mixture.

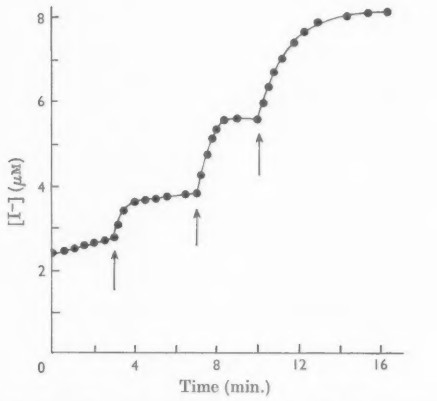

Fig. 2. Effect of xanthine on the aerobic reaction of xanthine oxidase (2·6μM) with iodoacetamide (12mM). At the times shown by the arrows 0·1ml. portions of xanthine (equivalent to a final concentration of 0·25mM) were added. The reaction medium (4ml. at 25°) was as follows (final concentrations): tris–maleate buffer, pH 7·2, 20mM; EDTA, 0·4mM; KI, 1μM; Tween 80, 0·02% (v/v). At the end of the experiment 14% of the xanthine-oxidase activity remained.

back to its original level. The rate could then be re-elevated by successive further additions of xanthine (Fig. 2). During the period of rapid iodide liberation enzymic activity was progressively lost; negligible activity was lost before xanthine was added. In control experiments, xanthine (final concn. 2·5mM) added to the reaction mixture in the absence of xanthine oxidase gave a small increase in the background rate of iodide liberation but this was in no way comparable with the effect produced in the presence of the enzyme.

Anaerobic experiments

The aerobic experiments showed that iodoacetamide reacts rapidly with xanthine oxidase only in the presence of the substrate, xanthine. Anaerobic studies were therefore undertaken to obtain further information and to confirm that oxygen was not involved in activating the enzyme towards the reagent.

Effect of xanthine oxidase concentration. Fig. 3 shows the time-course of iodide production at three different xanthine oxidase concentrations, other conditions remaining the same. Xanthine was added in excess at the time indicated and, as in the aerobic experiments, it caused an immediate increase in the rate of iodide production. After 10–15min. the rate decreased, but to a value higher than it had been before the addition of substrate. The rates of iodide production at all phases of the reaction increased as the enzyme concentration was raised. Similarly, the extent of the rapid phase of the reaction, which started when the xanthine was added, increased in proportion to the xanthine oxidase concentration.

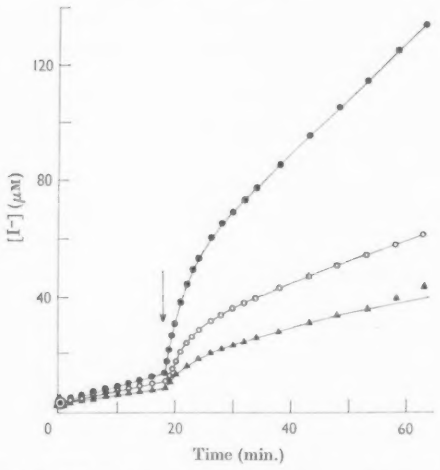

Fig. 3. Effect of xanthine oxidase concentration on the anaerobic reaction of xanthine oxidase with iodoacetamide (1·0mM). The final xanthine oxidase concentrations were: ▲, 8·5μM; ○, 17μM; ●, 34μM. After 18min. (indicated by an arrow) 0·1ml. of xanthine (equivalent to a final concentration of 0·44mM) was added to each sample. The reaction medium (2·3ml. at 25°) was as follows (final concentrations): tris–maleate buffer, pH7·4, 40mM; EDTA, 0·07mM; KI, 1μM; Tween 80, 0·02% (v/v).

Effect of iodoacetamide concentration. In general, in the anaerobic work, an iodoacetamide concentration of 1mM was employed. In one experiment this was increased to 4mM. The initial rate of the rapid phase of the reaction now too high for accurate measurement but it appeared to be at least 3 times as fast as that at the lower reagent concentration.

Effect of xanthine concentration. A series of experiments was performed with constant xanthine oxidase and iodoacetamide concentrations but with various xanthine concentrations. In Fig. 4(*a*) the curves of iodide production against time and of activity against time are presented for an experiment with excess of xanthine. Fig. 4(*a*) shows that the iodide production curve may be expressed as the sum of first-order and zero-order reactions which, when added, give a theoretical curve fitting the experimental data well. In this experiment the loss of activity went to completion and paralleled closely the first-order alkylation reaction. Fig. 4(*b*) shows a similar experiment at a lower xanthine concentration. Here, the activity loss was only partial but again it appeared to parallel the first-order reaction. In Fig. 5, the initial iodide rate and the extent of inactivation after 10min. are shown as a function of xanthine concentration for a series of experiments of the above type. Inactivation did not go to completion and initial iodide production rates were submaximal with less than 3–4mol.prop. of substrate.

Effect of pH. A series of experiments was carried out in the pH range 5·0–9·6, with an excess of xanthine. The stability of the enzyme in the reaction medium in the absence of xanthine at the two extremes of pH was first checked. At pH9·6 the activity apparently increased by 4% in 11min., whereas at pH5·0 it decreased by 8% in 10min. These changes may be regarded as negligible in comparison with those occuring in the presence of the substrate. Fig. 6 shows an experiment at pH9·6 in which the data have been analysed in the manner illustrated in Fig. 4(*a*). The rate of reaction was much higher under these conditions and, further, to obtain a fit to the iodide production curve it was necessary to assume that two first-order reactions, one fast and one slow, were proceeding, rather than a first-order and a slower zero-order reaction. Fig. 7 shows data obtained in a similar manner at a series

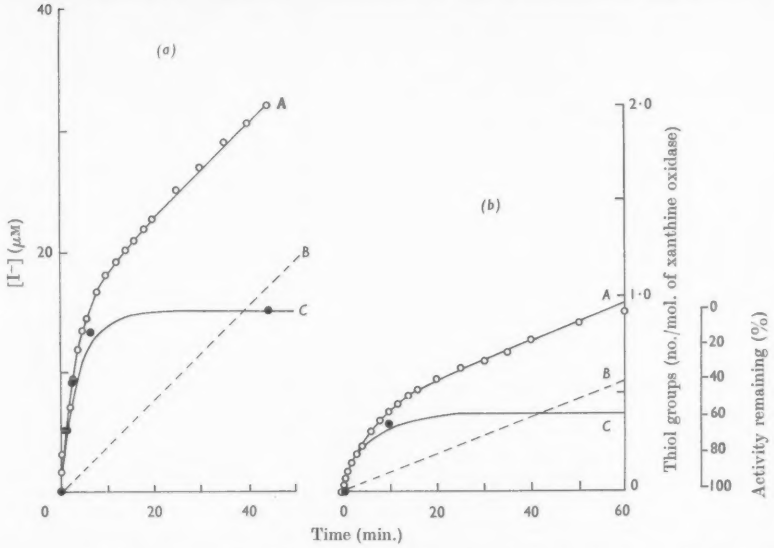

Fig. 4. Correlation between iodide production and loss of activity during anaerobic reaction of xanthine oxidase (16 μM) with iodoacetamide (1·0 mM) in the presence of 217 μM-xanthine (*a*) and of 11 μM-xanthine (*b*). The enzyme and substrate were mixed anaerobically at zero time. ○, Iodide concentration; ●, xanthine-oxidase activity. In (*a*) the theoretical line (*A*) through the experimental points (○) represents the sum of the broken line (*B*), a zero-order reaction with a velocity of 0·384 μM/min., and the third line (*C*), a first-order reaction with $k_1 = 0{\cdot}27\,\text{min.}^{-1}$ and $\Delta I_1 = 15{\cdot}0\,\mu$M. A similar summation is made in (*b*),where the broken line (*B*) is a zero-order reaction with a velocity of 0·150 μM/min. and the third line (*C*) a first-order reaction with $k_1 = 0{\cdot}17\,\text{min.}^{-1}$ and $\Delta I_1 = 6{\cdot}4\,\mu$M. The conditions were as given in Fig. 3 except that the concentration of EDTA was 0·03 mM.

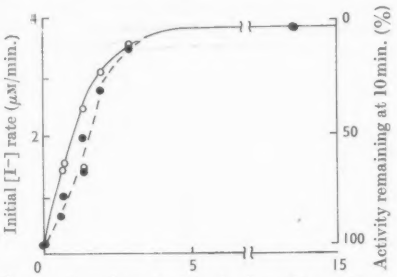

Fig. 5. Effect of xanthine concentration on the anaerobic reaction in a series of experiments similar to those in Figs. 4(*a*) and 4(*b*). ○, Xanthine-oxidase activity after 10 min. reaction; ●, initial rate of iodide liberation.

of pH values. Though the slower reaction did not deviate significantly from zero-order kinetics during a period of about 1 hr., except at pH 8·8 and 9·6, nevertheless, first-order velocity constants were calculated from the initial observed zero-order rates by assuming that the extent of the reaction was the same (cf. Fig. 6) at all pH values. Fig. 7 shows, as a function of pH, the velocity constant for the slow phase (k_2; curve *C*), the velocity constant for the fast phase (k_1; curve *A*), the extent of the fast phase (ΔI_1; curve *B*) and the velocity constant of the fast phase as calculated from activity measurements (k_a; curve *A*). (The activity measurements were made 3 min. after adding the substrate and it was assumed that complete inactivation occurred). The k_1 and k_a points both fall on curve *A*, indicating once again parallelism between the rapid phase of iodide production and the inactivation of the enzyme. The velocity constants of both phases of the reaction increased with increasing pH. So also, apparently, did the extent of the rapid phase reaction, which amounted to 0·7 mol. of iodide/mol. of enzyme at pH 5·0 and to 1·3 mol./mol. of enzyme at pH 9·6.

Reaction with uric acid and with phenylxanthine. With 12·8 mol.prop. of uric acid in place of xanthine,

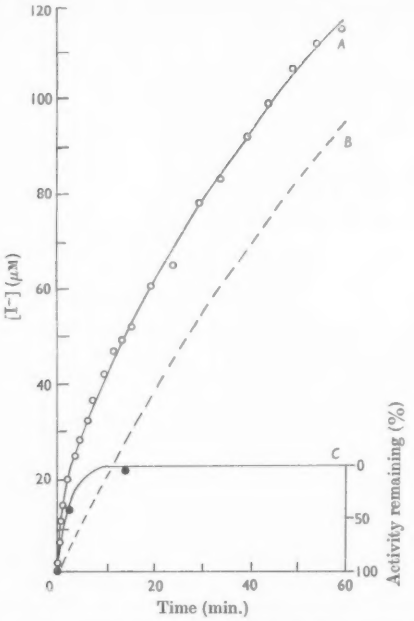

Fig. 6. Anaerobic reaction at pH 9·6 of 17 μM-xanthine oxidase with 1 mM-iodoacetamide and 217 μM-xanthine. ○, Iodide concentration; ●, xanthine-oxidase activity. The theoretical line (*A*) through the experimental points (○) represents the sum of the interrupted line (*B*), a first-order reaction with $k_2 = 0{\cdot}01\,\text{min.}^{-1}$ and $\Delta I_2 = 210\,\mu\text{M}$, and the third line (*C*), a first-order reaction with $k_1 = 0{\cdot}43\,\text{min.}^{-1}$ and $\Delta I_1 = 22{\cdot}6\,\mu\text{M}$. The conditions were as given in Fig. 4, except that 70 mM-carbonate buffer, pH 9·6, was used in place of tris–maleate buffer.

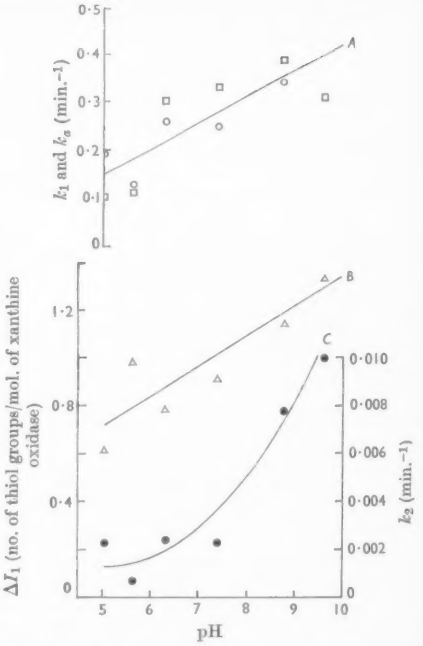

Fig. 7. Effect of pH on the two phases of the reaction. Experiments were carried out as described in Fig. 6 and the data were analysed as in Fig. 6 or Fig. 4(*a*). Curve *A*: ○, k_1 (min.$^{-1}$); □, k_a (min.$^{-1}$) calculated from activity measurements made after 3 min. reaction. Curve *B*: △, ΔI_1 (mol. of thiol group/mol. of xanthine oxidase). Curve *C*: ●, k_2 (min.$^{-1}$). The buffers used were as follows: pH 5·03, 70 mM-acetate; pH 9·62, 70 mM-carbonate; pH 7·38, 40 mM-tris–maleate; other pH values, 70 mM-tris–maleate.

no increase in the iodide production rate was found at pH 7·5. With 12·8 mol. prop. of 8-phenylxanthine at the same pH a brief rapid reaction was observed that corresponded to a ΔI_1 value of 0·06 mol. of iodide/mol. of xanthine oxidase.

Reaction with salicylaldehyde and with sodium dithionite. Fig. 8 gives the inactivation rates when salicylaldehyde or sodium dithionite was used to reduce the enzyme in place of xanthine. The aldehyde and purine substrates were equally effective, and dithionite gave a slightly higher rate of inactivation.

DISCUSSION

The results show clearly that reaction of approx. 1 mol. prop. of iodoacetamide with xanthine oxidase reduced with xanthine can lead to complete inactivation of the enzyme. Though it has not been proved that the reactive group in the reduced enzyme is a thiol it seems unlikely that any other group would react with iodoacetamide at a rate as high as that observed (cf. Gundlach, Stein & Moore, 1959). The presence of such a group in xanthine oxidase is in full agreement with the literature on the enzyme.

The exact number of thiol groups reacting/mol. of xanthine oxidase is not easy to determine from the stoicheiometry of the fast reaction (ΔI_1; Figs. 4*a* and 7), owing to a degree of uncertainty as to the true concentration of the enzyme. The concentration was determined from E_{450} but this measures both 'active' and 'inactive' enzyme (cf. Bray *et al.* 1961). According to Morell (1952) the latter may be slowly reduced by reduced 'active' xanthine oxidase. From Morell's data the velocity

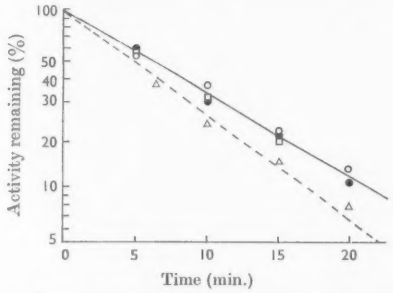

Fig. 8. Anaerobic inactivation of xanthine oxidase (20 μM) by iodoacetamide (1·0 mM) in the presence of xanthine (200 μM) (○ and □), salicyladehyde (190 μM) (●) and sodium dithionite (180 μM) (△). The reaction was carried out in 90 mM-tris–maleate buffer, pH 5·6, containing EDTA (0·04 mM). Other conditions were as given in Fig. 3. Activities are expressed as a percentage of that found before adding the substrate or dithionite. The continuous line corresponds to the first-order velocity constant, $k_a = 0{\cdot}108\,\text{min.}^{-1}$, and the broken line to $k_a = 0{\cdot}135\,\text{min.}^{-1}$.

of this reduction seems to be comparable with the velocity we find for the iodoacetamide-mediated inactivation process. Hence it is uncertain whether 'inactive' xanthine oxidase is participating in the reaction with iodoacetamide or not. However, the fact that two samples of xanthine oxidase with very different specific activities (see the Materials section) gave values for ΔI_1 as close as 0·9 and 1·0 mol. of thiol group/mol. of xanthine oxidase at pH 7·4 (Figs. 4*a* and 7 respectively) suggests that both 'active' and 'inactive' enzyme must be taking part and reacting indistinguishably. It is thus reasonable to infer that 'active' xanthine oxidase has 1 reactive thiol group/mol. Comparison with the work of Brumby *et al.* (1965) suggests that substrate reduction activates more thiol groups for reaction with phenylmercuric acetate than it does for reaction with iodoacetamide. Since these authors used the liver enzyme the difference could, of course, simply reflect differences between the liver and milk enzymes. However, mercurial compounds in general, unlike iodoacetamide, are very destructive reagents and reaction is frequently accompanied by gross changes in protein structure, as indeed the data of Brumby *et al.* (1965) show. A similar phenomenon has been observed for yeast alcohol dehydrogenase (cf. Hoch & Vallee, 1959; Rabin, Ruiz Cruz, Watts & Whitehead, 1964). It is therefore suggested that phenylmercuric acetate reacts with the thiol group that is sensitive to iodoacetamide but that in addition it reacts with several more thiol groups, as a consequence of disruption of the structure of the protein by the reagent.

Increasing the pH increases the velocity (k_1) of the rapid phase of the alkylation reaction (Fig. 7) but not to the large extent that would be expected for a freely-ionizing thiol group (Hollaway, Mathias & Rabin, 1964). Further, even at the highest pH values, the velocity of the reaction is still relatively low. Hence it is concluded (cf. Watts & Rabin, 1962; Rabin *et al.* 1964) that the thiol is modified by interaction with another group on the enzyme. Interaction of the thiol group with the substrate (or product) seems to be excluded, since the reaction rate (k_a) is identical with two different substrates and is only slightly different if the enzyme is reduced with sodium dithionite (Fig. 8; see below). The reason for the small but apparently significant increase in the number of groups reacting (ΔI_1) as the pH is raised is not clear.

The experiments of the titration type (Fig. 5), in which the xanthine concentration was varied, may be compared with electron-spin-resonance titrations of the enzyme reported by Ehrenberg & Bray (1965). They found that 4–5 mol.prop. of xanthine was required to obtain complete reduction of the enzyme. The present results suggest that about the same, or possibly a slightly lower, molar ratio of xanthine may be adequate for maximum sensitivity to iodoacetamide. In the presence of excess of xanthine the reaction appeared to be first-order with respect both to iodoacetamide and to xanthine oxidase.

It was not possible to measure iodide production when xanthine oxidase was reduced by salicylaldehyde or sodium dithionite, as these react with the silver–silver iodide electrode. Nevertheless, it is clear that they produce an effect on enzyme inactivation comparable with that given by xanthine (Fig. 8). It is concluded that the grouping reactive towards iodoacetamide is present in reduced xanthine oxidase itself and does not depend on the presence of bound substrate. The fact that sodium dithionite gave slightly more rapid inactivation than did the substrates might be due to side reactions or to a small measure of protection afforded by the substrates. Further indirect evidence that xanthine was simply reducing the enzyme was deduced from the fact that two non-reducing substances capable of interacting with the active centre failed to give any effect comparable with that produced by xanthine. Thus the reaction product, uric acid, had no detectable effect on iodide liberation. Similarly, 8-phenylxanthine, which is a competitive inhibitor of xanthine oxidase with K_i in the range 1–10 μM (A. J. Chisholm, unpublished work), gave only an extremely slight enhancement in the rate of iodide production and this might have been due to reducible impurities in the phenylxanthine sample.

The slow phase of the reaction of the enzyme with

iodoacetamide must reflect general alkylation of other groups, presumably including some of those groups that react with mercurials. Its velocity is higher in the reduced enzyme, after the rapid reaction is complete, than it is in the oxidized enzyme (Fig. 3). This indicates that steric changes must be caused in the enzyme, either by reduction or by the rapid alkylation reaction. The velocity of the slow reaction increases with increasing pH.

There is at present little evidence as to how a reactive thiol is involved in the catalytic reaction of xanthine oxidase. Fridovich & Handler (1958) postulated a metal–sulphur bond in the oxidized enzyme that, on uptake of a single electron supplied by the substrate, splits to the reduced metal and a thiol group. Attractive as such a mechanism may be, direct evidence for it seems to be lacking at the present time. More recently, Rajagopalan & Handler (1964) suggested that a molybdenum mercaptide group was present in the active centre of the closely related enzyme aldehyde oxidase. On the other hand, lipoyl dehydrogenase has been reported to contain an essential disulphide group in the oxidized enzyme that is reduced by two electrons from the substrate to a pair of thiol groups (Massey & Gibson, 1964). Such a mechanism, if it operated in xanthine oxidase, would require 2 thiol groups to appear on reduction (or 4 thiol groups, if 2 active centres/mol. are assumed). The fact that we find close to 1 thiol group/mol. argues against this, but the possibility that 2 thiol groups/mol. are involved is not completely excluded by our results and hence we are unable to distinguish between these mechanisms. A related possibility is that a disulphide group in the resting enzyme is split on reduction to give 1 free thiol group, the second sulphur atom then reacting with some other group in the enzyme, perhaps, for example, with quinquevalent molybdenum. If this secondary intramolecular reaction should be incomplete at high pH values, it would explain the variation of ΔI_1 with pH. It should be pointed out that the disulphide link postulated here must, from stoicheiometric considerations, be distinct from the disulphide groups proposed by Brumby *et al.* (1965) and which they suppose to be related to the iron and 'labile sulphide' of xanthine oxidase. Finally, the possibility cannot be excluded that the thiol group arises not as a direct consequence of reduction, as is implied in the above mechanisms, but as a result of steric changes in the enzyme.

We thank Mrs A. Chisholm for preparing the enzyme and Mr J. H. Trendall for his skilled construction of the anaerobic reaction vessel. R. C. B. thanks Professor F. Bergel, F.R.S., for his interest in this work, which was supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the Medical Research Council and the British Empire Cancer Campaign for Research, and by Public Health Service Research Grant no. CA-03188-08 from the National Cancer Institute, U.S. Public Health Service. Grants to University College London from the Central Research Fund for the purchase of the automatic titration apparatus and from the Wellcome Foundation for a Cary spectrophotometer are also gratefully acknowledged.

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An Investigation of Serum Lipoproteins and of their Contribution to Milk Fat in the Dairy Cow

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(*Received* 4 *May* 1965)

1. Tritium-labelled olive-oil triglycerides were introduced into the rumens of lactating cows and the specific activities of the lipids of milk and plasma and of serum lipoproteins were measured. 2. On treatment of serum with dextran sulphate it was found that the lipid of the precipitated β-lipoproteins consistently had a specific activity–time curve with a maximum comparable in value with, and occurring earlier than, that of the milk fat. 3. On fractionation of the lipids of these lipoproteins it was found that only the triglycerides and diglycerides had specific activity–time curves with maxima greater than that of milk fat, and on radioactivity data alone they are the only blood constituents studied that meet the requirements for being the precursor of milk fat. 4. From a consideration of abundances and the mean specific activities over the period of the experiment it is shown that the contribution of the diglycerides to the radioactivity in the milk fat must have been negligible and that only the triglycerides could have been responsible for all the radioactivity found in it. 5. Although no other fraction could alone have been responsible for all the radioactivity in the milk fat, at least one, the phospholipids, could have made some contribution. 6. It is calculated that 35–48% by weight of milk fat was derived from the β-lipoprotein triglycerides, according to whether the phospholipids made an improbably large contribution or none at all.

It was previously reported from this Laboratory (Glascock, Duncombe & Reinius, 1956) that when tritium-labelled stearic acid, either free or in the form of triglycerides, was fed to a lactating cow, up to 59% of the radioactivity was transferred to the milk fat. It was already known that milk fat is synthesized in the udder from small molecules such as acetate (Popják, French & Folley, 1951), but this work showed that it is also derived from lipid precursors, as, in fact, had been concluded many years earlier by Graham, Jones & Kay (1936). The specific activity of the mixed plasma lipids reached a maximum about 40hr. after dosing, whereas that of the milk fat did so at 23hr.; and the maximum specific activity of the milk fat was 5–10 times as great as that of the plasma lipids, which could not therefore be wholly or mainly its precursor (Glascock, McWeeny & Smith, 1958).

It was concluded that freshly absorbed fat circulates in the form of a compound that is selectively removed by the udder for conversion into milk fat. In these experiments it must have had a maximum specific activity greater than that of the milk fat and therefore more than 10 times as great as that of the mixed plasma lipids, in which its abundance must have been correspondingly low. The present work was designed to identify this fatty compound. Preliminary accounts of various parts of this work have already been published (Welch, Noble, Wright, Bishop & Glascock, 1963; Glascock, Welch, Bishop & Davies, 1965).

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EXPERIMENTAL

Chemicals

Solvents were all reagent grades. Ether was dried over sodium and redistilled. Hexane, benzene, chloroform, ethanol and methanol were distilled once. Toluene, for scintillation counting, was distilled three times. Diphenyloxazole was supplied by Thorn Electronics Ltd. Hyamine hydroxide was prepared from Hyamine 10X, supplied by Charles Lennig and Co. (Gt. Britain) Ltd., London, W.C. 1, by the method of Eisenberg (1958). Silicic acid for column chromatography was Mallinckrodt 100-mesh grade and supplied by Kodak Ltd., Kirkby, Liverpool. Merck Silica Gel G for thin-layer chromatography was supplied by Camlab (Glass) Ltd., Cambridge. Florisil (magnesium trisilicate) was 60–100 mesh and supplied by L. Light and Co. Ltd., Colnbrook, Bucks. Cellulose powder was Whatman standard grade. Purified phosphatide was a gift from the

Upjohn Company, Kalamazoo, Mich., U.S.A., which is gratefully acknowledged. Pitocin at 10 international oxytocin units/ml. was supplied by Parke, Davis and Co. Ltd., Hounslow, Middlesex. Dextran sulphate was clinical grade, supplied by Glaxo Laboratories Ltd., Greenford, Middlesex.

Dextran sulphate reagent. This consisted of a filtered mixture of 1 vol. of a 10% (w/v) solution of dextran sulphate and 5 vol. of M-$CaCl_2$ solution.

Tritium-labelled triglycerides. [^{3}H]Stearic acid was prepared as described by Glascock & Reinius (1956) by the catalytic addition on a micro-scale of tritium, in the chemical form of hydrogen gas, to elaidic acid. [^{3}H]Stearic acid thus prepared was usually pure white with m.p. 71° and a specific activity of about 200 mc/mg. The tritium is known to be unevenly distributed on the carbon chain and not specifically in the 9,10-positions (Glascock & Reinius, 1956).

A suitable total activity was transesterified with 0·5 g. of olive oil by the method of Bergström, Borgström & Rottenberg (1952). The product was purified by chromatography on silicic acid as described below for blood lipids.

Oil emulsion. The oil emulsion for oral administration was prepared as described by Meyer, Fansher, Schurr & Webster (1957); the purified labelled triglyceride was dissolved in 5 g. of olive oil together with 2·5 g. of monostearin and 100 mg. of purified phosphatide. The mixture was vigorously stirred in 100 ml. of boiling water for 10 min. by means of an MSE homogenizer, yielding a stable emulsion.

Experimental animals

Three separate experiments (numbered 62/1, 62/2 and 63/1) were carried out on Friesian cows of the Institute herd. Each animal was starved for 24 hr. before dosing and had a polythene cannula inserted in the right jugular vein to enable blood samples to be taken without disturbing her. During the experiment, which lasted up to 4 days, she received 4·5 kg. of hay and 2·7 kg. of concentrates twice daily (at 6·00 a.m. and 4·00 p.m.) with free access to water. After the injection of 2 ml. of Pitocin through the cannula, she was milked out completely just before dosing and when milk samples were taken. In Expts. 62/1 and 62/2 the labelled triglyceride was given by stomach tube in 100 ml. of emulsion followed by 100 ml. of unlabelled emulsion, making a total dose of 15 g. of fat. In Expt. 63/1 these quantities were halved and the emulsion was given by intraruminal injection through a previously inserted polythene cannula. The points on the graphs show the times at which blood samples were taken and the midpoints of the periods between milkings. Other experimental details are summarized in Table 1.

Treatment of samples

Milk. A 50 ml. portion was withdrawn from each sample and freeze-dried.

Separation of plasma and red cells. The samples of citrate-treated blood were centrifuged for 30 min. at 1400*g*. Plasma was separated, its volume was measured and it was freeze-dried. The red cells were suspended in 45 ml. of iso-osmotic solution and were centrifuged for 10 min. at 1400*g*, the supernatant was discarded, the procedure was repeated twice more and the cells were freeze-dried.

Standardization of lipoprotein precipitation. Before each experiment, the optimum amount of dextran sulphate reagent required to precipitate the β-liproteins was determined by titrating the serum with dextran sulphate reagent to maximum turbidity (Oncley, Walton & Cornwell, 1957).

Separation of lipoproteins. The method was based on that of Burstein & Samaille (1957). The samples of blood were allowed to stand for 12 hr. at 4° and they were then centrifuged for 30 min. at 1400*g*. Serum was aspirated from the cells, its volume was measured, the calculated amount of dextran sulphate reagent was added and the mixture left in the refrigerator at 4° for 12 hr. It was then allowed to warm to room temperature and centrifuged at 2800*g* for 1 hr. at 20°. The supernatant, which contained the α-lipoproteins and the unesterified fatty acids, was freeze-dried. The precipitated lipoproteins were redissolved in a solution of NaCl (5%, w/v) equivalent in volume to one-twentieth of the original volume of serum, and the lipoproteins were reprecipitated by the addition to that solution of 10 times its own volume of $CaCl_2$ solution (0·1 M). The mixture was stored at 4° for 2½ hr. and the lipoproteins were separated by centrifugation at 20° for 30 min. at 2800*g*. The supernatant solution was discarded.

Electrophoresis. This was carried out on Whatman 3MM paper for 5 hr. at a potential difference of 240 v in veronal buffer, pH 8·6 and *I* 0·05. Protein bands were stained with bromophenol blue and lipoprotein bands with Sudan Black B as recommended by Swahn (1953). On electrophoresis, whole serum gave four well-defined protein bands, which are identified as albumin, α-globulin, β-globulin and

Table 1. *Experimental animals*

Expt. no.	Date	Weight of animal (kg.)	Day of lactation	Milk yield (kg./day)	Total radioactivity given (mc)	Volumes of blood samples
62/1	February 1962	473	124	12	45·5	75 ml.* 125 ml.
62/2	October 1962	525	59	9	36	225 ml.
63/1	May 1963	605	241	13·6	46	225 ml. (jugular vein) 225 ml. (mammary vein)

* Taken into citrate for plasma separation.

γ-globulin (which remained at the origin or moved slightly behind it). The supernatant, after separation of the lipoprotein–dextran sulphate precipitate, gave a lipoprotein band lying between the α-globulin and the albumin, and in conformity with the nomenclature used by workers on human serum (e.g. Smith, 1960) we provisionally call this fraction α-lipoprotein. The redissolved lipoprotein–dextran sulphate precipitate gave a band migrating close to the β-globulin and another remaining at the origin with diffuse staining in between. For convenience we call the whole of the material precipitated by dextran sulphate β-lipoprotein.

Extraction of lipids. Freeze-dried plasma, α-lipoproteins, milk and red cells were shaken for 3hr. with a volume of chloroform–methanol (2:1, v/v) equal to that of the original fresh sample. A mixture of chloroform–methanol and red cells gave a rubbery mass which could be avoided by the addition of one-third of a volume of ethanol to the solvent mixture. The mixture was filtered on a Buchner funnel, and the residue was washed and then shaken for a further 24hr. with the same volume of solvent. The suspension was again filtered and the procedure repeated. Finally, the residue was submitted to Soxhlet extraction with the same solvent mixture for 3hr. The extracts were combined, concentrated to 20ml. under reduced pressure and freed from protein by passing through a column of cellulose powder, as described by Böttcher, Woodford, Boelsma-Van Houte & Van Gent (1959).

The extracts of plasma and milk were concentrated to small volume by distillation under reduced pressure, made up to 100ml. with chloroform–methanol (2:1, v/v) and stored at −10°. The extract of α-lipoproteins was concentrated under reduced pressure almost to dryness and further purified by redissolving in 100ml. of chloroform–methanol (2:1, v/v) and partitioning with one-quarter of its volume of water as described by Folch, Lees & Sloane-Stanley (1957). The lower phase was concentrated to small volume under reduced pressure, 50ml. of benzene was added and the mixture was distilled almost to dryness to remove water. This procedure was repeated and the residue was finally dissolved in hexane, made up to 100ml. and stored at −10°.

β-Lipoproteins. To the precipitated β-lipoproteins was added 25ml. of chloroform–methanol (2:1, v/v), and the mixture was filtered through a Soxhlet thimble (7·5cm. ×2·5cm.). The small residue was extracted in a Soxhlet apparatus for 3hr. with the same solvent. The extract was concentrated nearly to dryness, a few millilitres of chloroform–methanol (2:1, v/v) were added and the solution was combined with the first extract and made up to approx. 80ml. It was then partitioned with one-quarter of its volume of water and the procedure continued as described for the α-lipoproteins.

Fractionation of lipids

The technique used for fractionation on columns of silicic acid was basically that of Barron & Hanahan (1958). This permitted the separation of all main classes of lipids except the free fatty acids, which were separated on a Florisil column as described by Carroll (1961). A maximum of 50mg. of mixed lipids was applied to a column prepared from 3g. of activated silicic acid and eluted by a stepwise procedure in which the volumes of various solvents required were determined by reference to a radiochromatogram. This was previously prepared by eluting one sample and measuring the total radioactivity in 5ml. fractions; elution with each solvent was continued until the radioactivity diminished to a negligible level. The volumes required did not greatly differ from experiment to experiment.

Thin-layer chromatography. Thin-layer chromatography of spots containing not more than 200μg. of mixed lipids was done on layers of Merck Silica Gel G, 250μ thick. The chromatograms were developed for about 30min. in ether–hexane–acetic acid (10:90:1, by vol., for separation of sterol esters, sterols, triglycerides and fatty acids, or 50:50:1, by vol., for separation of monoglycerides, diglycerides and triglycerides; phospholipids remain at the origin in both systems). Spots were stained with dodecamolybdophosphoric acid [10% (w/v) in 95% (v/v) ethanol].

Measurement of radioactivity. Specific radioactivity was determined by the liquid-scintillation counting of weighed samples in an Ecko single-channel scintillation counter. The scintillation system was diphenyloxazole (3g./l.) in a 9:1 (v/v) mixture of toluene and ethanol. In Expts. 62/1 and 62/2 samples of total lipids were assayed in duplicate but only single samples of the fractions were assayed. In Expt. 63/1 most samples were assayed in duplicate.

Titration and counting of diglyceride fatty acids. Thin-layer chromatography showed that the diglycerides obtained in Expt. 63/1 were contaminated with sterols. In this experiment only therefore, the diglycerides were saponified and the fatty acids were liberated, extracted, titrated and counted.

Monitoring of samples. In Expts. 62/2 and 63/1 the radioactivity of samples of blood and milk was measured as soon as possible after taking them, to ensure that sampling continued until specific activities passed their maximum. Serum was counted directly by adding 0·1ml. to a mixture of 5ml. of phosphor solution and 1ml. of Hyamine hydroxide. Milk was diluted with an equal volume of water and 0·2ml. added to the same mixture.

RESULTS

Experiment 62/1. Fig. 1(*a*) shows the specific activity–time curves of the total mixed lipids of milk, plasma, α- and β-lipoproteins and blood cells. The collection of samples ceased before the specific activity of the milk fat reached a maximum and in this respect the experiment was anomalous. In all other experiments the maximum had been reached at about 23hr., but in this one the specific activity of the milk fat was still rising 36hr. after dosing. Until it reached its maximum at 27hr. the specific activity of the β-lipoprotein lipids was about the same as that of the milk fat. The specific activity of the α-lipoprotein lipids, however, was even lower than that of the total plasma lipids. This was to be expected since the β-lipoproteins, whose lipids had a high specific activity, had been removed. The specific activity of the lipids of the red blood cells was lower than that of any of the other samples collected, showing that they make little or no contribution to the radioactivity in the milk fat.

The specific activity of none of the fractions of plasma lipids reached values even approaching the

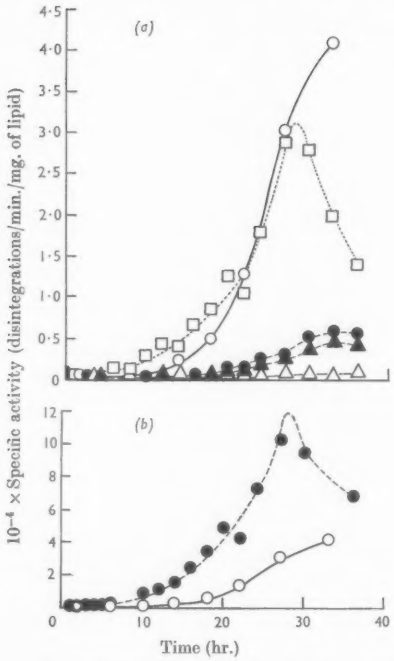

Fig. 1. Expt. 62/1: specific activity of milk and blood lipids of a cow after administration of [^{3}H]stearic acid as olive-oil triglycerides. (*a*) Mixed lipids of milk (○), plasma (●), serum β-lipoproteins (□), serum α-lipoproteins (▲) and red cells (△); (*b*) milk mixed lipids (○) and serum β-lipoprotein triglycerides (●).

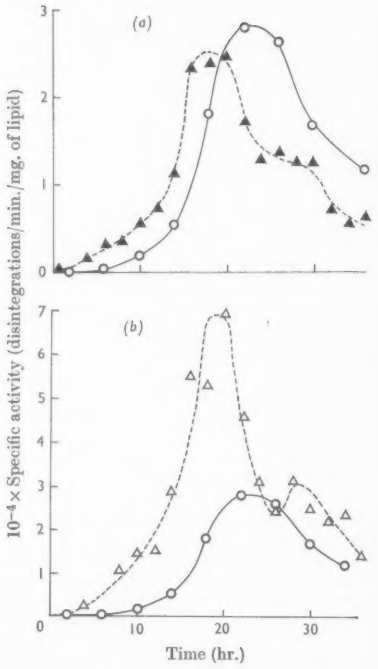

Fig. 2. Expt. 62/2: specific activity of milk and blood lipids of a cow after administration of [^{3}H]stearic acid as olive-oil triglycerides. (*a*) Milk mixed lipids (○) and serum β-lipoprotein mixed lipids (▲); (*b*) milk mixed lipids (○) and serum β-lipoprotein triglycerides (△).

highest observed in the samples of milk fat. The finding in a previous experiment (Glascock & Wright, 1962) that the specific activity of an unidentified fraction reached a maximum earlier and greater than that of the milk fat was therefore not confirmed.

The specific activity–time curves of milk fat and β-lipoprotein triglycerides are shown in Fig. 1(*b*). These triglycerides were the only fraction whose maximum specific activity was greater than that of the milk fat at 36hr., when the shape of the curve suggests that it was nearing its maximum. The maximum specific activity of the β-lipoprotein triglycerides occurred at 27hr., when it was 2½ times as great as that of the milk fat at 36hr. The fall in specific activity observed in the β-lipoprotein mixed lipids between 20 and 22hr. is also shown by the triglycerides, and it is therefore possible that the curve should be drawn through both these points instead of as shown in Fig. 1(*b*).

Thin-layer chromatography in a later experiment (63/1) showed that the diglycerides, as eluted from the silicic acid column, were often heavily contaminated with sterols. It is therefore possible in this experiment that the diglycerides had specific activities as high as those of the triglycerides.

Experiment 62/2. The specific activities of milk fat and of β-lipoprotein mixed lipids had about the same value at their maxima, as shown in Fig. 2(*a*). The maximum specific activity of the β-lipoprotein lipids occurred earlier than that of the milk fat, which was at the usual time of 24hr. In this experiment, as in Expt. 62/1, the maximum specific activities of all fractions of β-lipoprotein lipids except the triglycerides were less than that of the milk fat. The maximum specific activity of the triglycerides was 2½ times as great as that of the milk fat, as shown in Fig. 2(*b*). It is possible in this experiment, as in Expt. 62/1, that the diglycerides, as separated, were contaminated with sterols and

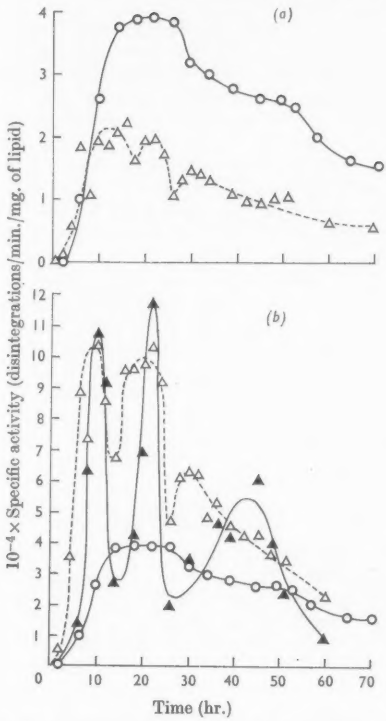

Fig. 3. Expt. 63/1: specific activity of milk and blood lipids of a cow after administration of [^{3}H]stearic acid as olive-oil triglycerides. (*a*) Milk mixed lipids (○) and serum β-lipoprotein mixed lipids (△); (*b*) milk mixed lipids (○), serum β-lipoprotein triglycerides (△) and diglycerides (▲).

had a specific activity about the same as that of the triglycerides.

Experiment 63/1. Although the results of this experiment were substantially in agreement with those of Expts. 62/1 and 62/2 there were some differences in detail, as shown in Fig. 3. The maximum in the specific activity–time curve of the milk fat is less sharp than in the earlier experiments and is almost flat over the period 14–26hr. The maximum specific activity of the mixed lipids of the β-lipoproteins was considerably lower than that of the milk fat and the curve showed three maxima, of which the first, at 12hr. is less well-defined by the experimental points than the other two at 22 and 30hr. It might be thought that these maxima were artifacts arising from experimental error in the determination of specific activities, particularly since their existence depends so much on the validity of the minima occurring at 18 and 26hr. If these two points are regarded as aberrant then a curve with a single maximum at about 16hr. can be drawn without any of the other experimental points lying very far off it. Such a curve was in fact published in a preliminary account of this work (Glascock *et al.* 1965). However, the two minima are the result of duplicate analyses in very good agreement and there appears to be no justification for discarding them. Further, the specific activity–time curves of the diglycerides and triglycerides, which were the fractions of greatest specific activity contained in these mixed lipids, also showed three strongly marked maxima (Fig. 3*b*). It thus appears that the time-course of the specific activity of the mixed lipids of the β-lipoproteins is best represented by the curve given in Fig. 3(*a*).

In Fig. 3(*b*) are shown the specific activity–time curves of the β-lipoprotein diglycerides and triglycerides and of the milk fat. In previous experiments only the triglycerides had a maximum specific activity greater than that of the milk fat. As noted above, however, it was found that the diglycerides in this experiment were contaminated with sterols. Assay of the fatty acids extracted from the hydrolysed diglyceride fraction showed that it had a specific activity–time curve similar to that of the triglycerides.

Both of these fractions show maxima that may also have existed in earlier experiments, as shown by the points that have been plotted but through which it was not thought justifiable to draw the curve. The maxima are also much more pronounced than they could have been in those experiments, which may be because the curves more closely reflect the time-course of specific activities as a result of improved techniques. It is also possible that a different method of administration of the labelled oil produced a different pattern of absorption.

DISCUSSION

The origin of the maxima in the specific activity–time curves of some of the lipids of the serum β-lipoproteins is at present a matter for conjecture. Absorption from one or more of the compartments of the stomach would result in the lipids' being carried to the liver. This could delay their appearance in peripheral blood whereas absorption from the intestine into the lymphatic system would not. The simultaneous operation of these processes might well produce the complex specific activity–time curves actually observed in the peripheral blood lipids.

In a precursor–product relationship the precursor reaches a maximum specific activity both greater and earlier than that of its product (Zilversmit, Entenman & Fishler, 1943). If it is the sole precursor and if the observations continue until

the bulk of the tracer has been swept through the system, the mean specific activities of precursor and product tend to equality. If there is another and unlabelled precursor the mean specific activity of the product is less than that of the labelled precursor in proportion to the degree of dilution of isotope produced by the unlabelled precursor.

A precursor–product relationship can be inferred from the curves for the β-lipoprotein triglycerides and milk fat of Expt. 62/2 (Fig. 2*b*), and also from Expt. 62/1 (Fig. 1*b*) as similar curves would almost certainly have been obtained had it been allowed to continue long enough. This inference is not invalidated by the brief minimum in the curve at 14hr. in Expt. 63/1 (Fig. 3*b*) since this would merely have had the effect of flattening the maximum of the product curve, as was, in fact, observed in the curve of the milk fat. Similarly the third smaller maximum would be expected to produce a change in the slope of the new descending product curve, and this again was observed.

Expt. 63/1 was the only one in which observations were continued long enough for most of the tracer to be swept through the system and hence which permits an estimate of the contribution of precursor to product from a comparison of specific activities. Such a calculation involves the assumption that the precursor was responsible for all the radioactivity in the product.

Graphical integration between the limits of 0 and 72hr. of the curves shown in Fig. 3(*b*) gave a mean specific activity for the milk fat of $2{\cdot}25 \times 10^4$ disintegrations/min./mg., which is in good agreement with the value of $2{\cdot}27 \times 10^4$ disintegrations/min./mg. observed. Similarly the mean specific activities of the diglycerides and triglycerides were found to be $3{\cdot}57 \times 10^4$ and $4{\cdot}86 \times 10^4$ disintegrations/min./mg. These values, together with those of the other β-lipoprotein lipids, are shown in Table 2.

The diglycerides and triglycerides are the only fractions whose maximum and mean specific activities were both greater than that of the milk fat and the only ones therefore that could have been responsible, on the evidence of specific activities alone, for all the radioactivity in the milk fat. The diglycerides can be excluded as a sole precursor, or even as an appreciable co-precursor, however, because of their low abundance, which (Table 2) was only about 1mg./l. of whole blood. The probable upper limit for the rate of blood flow through the udder is 171./min. [Glascock & Wright (1962) suggest a range 7–171./min.]. Complete removal of these diglycerides during passage through the udder could therefore provide a rate of secretion into milk of not more than 17mg./min. for fat and $6{\cdot}1 \times 10^5$ disintegrations/min./min. for radioactivity. These are only 6 and 10% respectively of the observed mean rates of secretion into milk, which were 275mg./min. for fat and $6{\cdot}25 \times 10^6$ disintegrations/min./min. for radioactivity. Further, provisional results indicate that in fact very little, if any, of the diglycerides was actually removed during passage through the udder.

The triglycerides are the only other possible single source of radioactivity in the milk fat and a comparison of mean specific activities gives a value of 46·3% as the fraction by weight of total milk fat derived from them. Extrapolation shows that this value would not have been more than 48% even if the experiment had been continued until all specific activities were virtually zero. The mean rate of secretion of milk fat was 275mg./min., and if 48% of this were derived from the triglycerides they must have been removed from the blood at a rate of 133mg./min. With only a 50% arteriovenous difference (say 10mg./l.) such a rate of loss would require a rate of blood flow through the udder of 131./min., which is well within the estimated range. The contribution of the β-lipoprotein

Table 2. *Concentrations and mean specific activities of radioactive lipid components of the jugular serum β-lipoproteins in the lactating cow (Expt. 63/1)*

Concentrations expressed as mg./100mg. of total lipids are given as means ± S.E.M. with the numbers of determinations in parentheses. Mean specific activities were obtained by graphical integration between the limits 0 and 72hr. of specific activity–time curves.

Component	Concentration (mg./100mg. of total lipids)	Concentration (mg./l. of serum)	$10^{-4} \times$ Mean specific activity (disintegrations/min./mg.)
Unidentified fraction	1·14 ± 0·07 (30)	4·43	0·48
Free fatty acids	2·52 ± 0·21 (36)	9·80	0·53
Monoglycerides	1·17 ± 0·06 (32)	4·55	0·30
Diglycerides	0·50 ± 0·07 (15)	1·94	3·57
Triglycerides	10·07 ± 0·61 (43)	39·15	4·86
Phospholipids	24·44 ± 0·86 (33)	95·02	0·80
Sterol esters	41·63 ± 0·88 (31)	161·86	0·10

triglycerides calculated from specific-activity data is therefore consistent with other known facts. The value would, however, be too high if other labelled lipids contributed to the radioactivity in the milk fat, and of the present results it cannot be said with certainty that they did not do so at all. The contribution of the lipids of low abundance (monoglycerides, unesterified fatty acids and the unidentified fraction) can be shown to be negligible, however, on the same grounds as that of the diglycerides: at any reasonable rate of blood flow through the udder even a 100% arteriovenous difference would have provided an insignificant fraction of the radioactivity actually found in the milk fat; and, further, preliminary results indicate that the arteriovenous differences for all these fractions was much less than 100%.

Of the two β-lipoprotein lipids of high abundance the sterol esters can have made no appreciable contribution to the radioactivity in the milk fat because their mean specific activity was so low compared with that of the triglycerides. The mean specific activity of the phospholipids, however, was one-sixth that of the triglycerides and some of the radioactivity in the milk fat might therefore have been derived from them. It is calculated that if the phospholipids were the only other precursor the radioactivity in the milk fat could not be accounted for by a contribution of less than 35% by weight of triglycerides. In this extreme and unlikely case, therefore, the real contribution of the triglycerides would be three-quarters of that calculated. It therefore seems safe to say that although a value of 48% is a maximum the true value for the proportion of milk fat derived from the β-lipoprotein triglycerides is unlikely to be much less.

Evans, Patton & McCarthy (1961) have shown that the lipoproteins migrating with approximately the same electrophoretic mobility as the β-globulins are those of low density ($d < 1{\cdot}063$); and Smith (1960) states that the lipid-containing material remaining at the origin on electrophoresis of human serum consists of lipoproteins of very low density in the form of particles greater in diameter than 1000Å and of chylomicrons (diameter 5000Å). It thus appears that the material precipitated with dextran sulphate in our work consists of lipoproteins of low density and, insofar as they exist in the cow, of chylomicrons. The results reported here are therefore in qualitative agreement with those of Barry, Bartley, Linzell & Robinson (1963), who found a substantial arteriovenous difference in the triglyceride fatty acids of chylomicrons and low-density lipoproteins ($d < 1{\cdot}019$) across the udder of the lactating goat. These workers also found that the arteriovenous differences in stearic acid and palmitic acid combined in these triglycerides were proportionately equal, indicating that there is no selective use or rejection of stearic acid relative to the other long-chain fatty acids. The assumption in our work that the [^{3}H]stearic acid effectively labels all the long-chain fatty acids of the β-lipoprotein triglycerides and is not selectively used by the udder for secretion into milk is therefore probably justified.

That circulating triglyceride can give rise to milk fat was demonstrated in this Laboratory as long ago as 1957 (Glascock *et al.* 1958) by the collection of radioactive milk fat after the injection of tritium-labelled olive-oil triglycerides into a lactating cow. This has been elegantly confirmed by Lascelles, Hardwick, Linzell & Mepham (1964), who injected chylomicrons, labelled *in vitro* with [^{3}H]stearic acid, into a lactating goat and showed that there was an efficient transfer of radioactivity into milk fat. The present work, which shows that only a small proportion of the total circulating triglycerides is used for milk-fat synthesis, is therefore in agreement with those results also.

We thank Dr A. T. Cowie and Dr J. Barna for the insertion of cannulas and other minor surgery, Dr Barna holding at the time a Fellowship of the International Atomic Energy Agency. We also thank Mr B. Brown, B.Sc., for advice and help in the management of animals, Mr S. H. Phillips for help with radioactive assay, and Miss D. Bosher, Miss J. Riley, Miss J. Sutton and Mr M. Cooney for technical assistance. E. W. W. was the holder of an F. A. O. André Meyer Fellowship. The work also received support, through grants to the Institute, from the Agricultural Research Council and the International Atomic Energy Agency, which is gratefully acknowledged.

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The Presence of Phosphatidylcholine in Neurons Isolated from the Lateral Vestibular Nucleus of Ox Brain

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(*Received* 9 *July* 1965)

1. Lipid was extracted from neurons isolated from the lateral vestibular nucleus of ox brain and examined by two-dimensional thin-layer chromatography. 2. The major lipids found were phosphatidylethanolamine and its plasmalogen, phosphatidylcholine, phosphatidylserine and cholesterol.

Since phosphatidylcholine has been reported as a major constituent of lipids from various fractions and subcellular particles of brain (Davison & Graham-Wolfaard, 1964; Eichberg, Whittaker & Dawson, 1964) it was surprising that we were unable to detect any appreciable amount in the lipids of neurons isolated from the lateral vestibular nuclei of ox brains (Roots & Johnston, 1965*a*). Though this finding could be an indication that phosphatidylcholine is largely a component of glia and myelin it could also have been due either to a peculiarity of the particular neurons studied or to a hitherto unrecognized analytical artifact. It occurred to us that the intracellular pigment, lipofuscin (Bondareff, 1964), was concentrated in the lipid extracted from pure samples of neurons and that the high proportion of this complex may have interfered with the normal thin-layer chromatographic migration patterns of the polar lipids. Previously we have found that, because of the proportion and nature of an accompanying pigmented complex, the lipids from the chloragosomes of the earthworm (*Lumbricus terrestris* L.) fail to separate into homogeneous fractions when subjected to various chromatographic procedures (Roots & Johnston, 1965*b*). Recently we achieved separation of these lipids by the use of two-dimensional thin-layer chromatography. We have therefore re-examined neuronal lipids by using this technique.

MATERIALS AND METHODS

Material. Neurons were isolated from the lateral vestibular nuclei of fresh ox brains by the method described by Roots & Johnston (1964). From each batch of brains approx. 1000 cells were collected and placed in Ringer–Locke solution in glass cavity slides. The solution was withdrawn and the cells were immediately frozen and stored at $-25°$.

Extraction of lipid. The lipid was extracted at room temperature with $3\times 100\mu$l. of chloroform–methanol (2:1, v/v) per slide. Three separate samples, the combined extracts from 5380, 2125 and 3600 neurons respectively, were examined. The solvent was removed under vacuum and the dry lipid was transferred to glass tubes (5mm. $\times$ 8mm.) by redissolving it in approx. 200μl. of the same solvent, which was then removed under a stream of O_2-free N_2. The lipid was examined immediately or stored only overnight under N_2 at $-10°$.

Thin-layer chromatography. Two-dimensional thin-layer chromatography was carried out on 3in. $\times$ 3in. plates with Silica Gel G as the adsorbent. Two or three platings were obtained from each lipid sample according to the size of the neuron collection (i.e. approx. 10–35μg. per plate). The plates were developed in the first direction with chloroform–methanol–water (65:25:4, by vol.) and in the second direction with butan-1-ol–acetic acid–water (6:2:2, by vol.) (Rouser, Galli, Lieber, Blank & Privett, 1964). In addition to the use of acid sprays and iodine vapour to reveal all lipids, plates were sprayed with 2,4-dinitrophenylhydrazine to reveal plasmalogens (Reitsema, 1954), ninhydrin to detect phospholipids containing free amino groups and a modified Dragendorf reagent to detect choline-containing lipids (Wagner, Hörhammer & Wolff, 1961). Several mixtures of phosphoglycerides and cholesterol were used to check the resolution and limits of detection. The lipids used were synthetic dipalmitoylphosphatidylcholine, dipalmitoylphosphatidylethanolamine (both from Sigma Chemical Co., St Louis, Mo., U.S.A.), phosphatidylserine (from ox brain; Koch–Light Laboratories Ltd., Colnbrook, Bucks.) and cholesterol (British Drug Houses Ltd., Poole, Dorset). In preference to the synthetic disaturated compounds phosphatidylethanolamine (from ox brain) and phosphatidylcholine (from egg) were used in some mixtures (Koch–Light Laboratories Ltd.). The relative proportion of the lipids was varied in each mixture and 10–40μg. samples were plated. The lipids were always well separated and it was possible to detect $0{\cdot}5\mu$g. of any lipid by using acid sprays or iodine vapour. The ninhydrin spray required $0{\cdot}5$–$1{\cdot}0\mu$g. and the Dragendorf reagent at least $7{\cdot}0\mu$g. of the appropriate compounds.

During collection of the neurons and subsequent extraction and treatment of the lipid, Perspex covers were used to protect against airborne contamination. The solvents used were either redistilled Reagent or AnalaR grades,

except methanol, which was Spectrosol grade (Hopkin and Williams Ltd., Chadwell Heath, Essex). All manipulations of the lipid including plating for thin-layer chromatography were carried out under an atmosphere of N_2.

RESULTS

Two-dimensional thin-layer chromatograms of the neuronal lipids showed phosphatidylcholine to be one of the major constituents. It was identifiable only by its R_F when smaller samples were used (10–25 μg.), and when larger samples were employed (25–35 μg.) by its positive reaction to the Dragendorf reagent. The other major lipids were phosphatidylethanolamine, phosphatidylserine and cholesterol. The first two of these were identified by their R_F values and their positive reaction to the ninhydrin reagent. The phosphatidylethanolamine spot also gave a positive reaction to 2,4-dinitrophenylhydrazine reagent, indicating that part of this lipid is present as the plasmalogen. The cholesterol spot at the second solvent front was obscured by the large amount of lipofuscin. However, on heating the plate after spraying with 50% (w/v) sulphuric acid the cholesterol was clearly visible and underwent the typical colour changes from blue to a reddish purple before charring. Two other spots could not be positively identified. These components appeared only when 25–35 μg. samples were used. One had a similar migration pattern to that of sphingomyelin but its R_F was not identical and it did not give a positive reaction to the Dragendorf reagent. The other gave a weak positive reaction to the ninhydrin reagent. It is probable that these spots are breakdown products that appeared in spite of the use of nitrogen to protect the lipids from oxidation.

In agreement with our preliminary results we found no evidence of cerebrosides in neuron lipid.

DISCUSSION

The separation of phosphatidylserine from phosphatidylethanolamine and phosphatidylcholine was one of the earlier difficulties of thin-layer chromatography. This problem was discussed by Skipski, Peterson & Barclay (1962) and by Horrocks (1963), who described procedures for successful separation. By using standard mixtures and the lipids of whole ox brain we found that unidimensional thin-layer chromatography on 'basic' plates (Skipski *et al.* 1962) gave good separation. However, it is now evident that our use of this procedure for 10–15 μg. of neuronal lipid did not give complete separation, the phosphatidylcholine and phosphatidylserine having migrated together. Since there was insufficient of the former constituent to react to the Dragendorf reagent but sufficient of the latter to give positive reaction to ninhydrin, the phosphatidylcholine remained undetected.

Pelick, Wilson, Miller, Angeloni & Steim (1965) have described various ways in which erroneous conclusions can be drawn from thin-layer chromatography; however, failure to separate constituents owing to a relatively high concentration of a lipid–pigment complex such as lipofuscin does not appear to have been encountered previously.

This work was supported by a grant from the Nuffield Foundation.

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Biochem. J. (1966) **98**, 159

A Function of the Golgi Apparatus in Polysaccharide Synthesis and Transport in the Root-Cap Cells of Wheat

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(*Received* 20 *May* 1965)

1. A radioautographic study of the cells of the root tips of wheat incubated with D-[1- or 6-^{3}H]glucose has shown that labelled material is formed in the golgi apparatus of the root-cap cells. This material passed to the vesicles associated with the golgi bodies and then moved through the cytoplasm across the plasmalemma and was incorporated into the cell wall and slime layer of the tissue. 2. Analysis of the labelled material extracted from the root tips showed that the bulk of the radioactive material was polysaccharide; there were relatively small amounts of labelled lipids and protein in the tissue. 3. Starch was formed from the exogenous labelled glucose and it was located in the plastids of the cell. The synthesis of starch depended on the metabolic activity of the cells, which varied with the position of the cell in the various tissues of the root tip and with the amount of the exogenous glucose. 4. Isolation of the radioactive polysaccharides from the root tip incubated in the radioactive glucose has shown that the glucose was very rapidly incorporated into the galactosyl residues of the polymers. 5. Analysis of the radioactive polysaccharides has indicated that the material transported in the golgi vesicles is probably pectic substance. 6. A scheme for the synthesis of the storage and wall polysaccharides by separate routes and their location within the cell has been put forward.

During the last 5 years there have been many indications that part of the synthesis of cell-wall material in plants takes place away from the site of wall formation and that the material is subsequently transported in vesicles to the wall where it is incorporated across the plasmalemma (cell membrane) by a process of pinocytosis (Northcote, 1962). During pinocytosis the membrane of the vesicle fuses and becomes continuous with the plasmalemma, and the content of the vesicle is passed out of the cell without any breakage of the cell membrane. This evidence is based on observations with the electron microscope, when it is seen that the golgi apparatus in particular seems to produce both the vesicles and the wall material. However, no direct evidence has yet been presented for this process. The golgi apparatus of plant cells is an organelle consisting of a stack of about six cisternae each bounded by a unit membrane and each approx. 0·6–1μ across. These cisternae are arranged on top of one another like a pile of plates.

The work described below has attempted two correlated lines of investigation: (*a*) a radioautographic study, in which 'pulses' of radioactive labelled glucose were applied to growing root tips, and the sequence of the movement of the label within the cell after various periods was followed by means of electron microscopy; (*b*) a direct chemical isolation and analysis of the resultant labelled material that had been incorporated into the wall and that was detected by the radioautographic investigation.

MATERIAL AND METHODS

Seedlings. Wheat seeds (*Triticum vulgare*) were soaked in water overnight, washed and germinated on damp filter paper. When the roots were about 1cm. long they were washed gently, blotted on filter paper and used for the experiments.

Chemical fractionation of root tips. The tissue was heated with 3% (w/v) NaOH (1ml.) for 1hr. in a boiling-water bath and the solution was separated from the insoluble material by a fine pipette. The extraction was repeated and the tissue was washed twice with boiling water (1ml.). The alkali solution and washings were combined. The insoluble residue represented the α-cellulose fraction.

The solution was dialysed against two changes of water (each 10l.) overnight at 4°, and then 1ml. of an α-amylase preparation (Olaitan & Northcote, 1962) was added to the non-diffusible material in solution and it was left for 6hr. at room temperature. The activity of the α-amylase solution was checked against a 1% (w/v) solution of soluble starch that reached the achromic point with iodine in 2hr. at room temperature.

The solution was redialysed against two changes of water

(each 10l.) at 4° for 24hr. and the solution inside the dialysis sac was freeze-dried. The residue represented the hemicellulose fraction.

Hydrolysis of polysaccharides. The polysaccharides were dissolved in 1 or 2 drops of H_2SO_4 (72%, w/v) at room temperature and the solution was diluted to 3% H_2SO_4 and autoclaved at 120° for 1hr. The solution was neutralized with $BaCO_3$ and filtered and the residue was well washed. The filtrate and washings were combined, deionized with Amberlite IR-120 (H^+ form) (British Drug Houses Ltd., Poole, Dorset) and freeze-dried.

Hydrolysis of whole root tips for amino acids. Dried lipid-extracted root tips (six) (see below) were hydrolysed in 6N-HCl for 24hr. in a sealed tube at 100°. The solution was evaporated to dryness under reduced pressure at 100° and re-evaporated four times with 5ml. of water. The dried residue was dissolved in 0·2ml. of water and applied to an electrophoretogram.

Lipid extraction. Dried root tips (six) were extracted with boiling dry methanol (4ml.) for 6hr. The roots were washed in methanol, and the extract and washings were combined and evaporated to dryness. The root tips were redried in a vacuum desiccator and then further extracted with boiling dry diethyl ether (5ml.) for 6hr. The roots were washed in ether, and the extract and washings were combined and evaporated to dryness.

Chromatography. Neutral monosaccharides present in polysaccharide hydrolysates were separated by descending paper chromatography on Whatman no. 1 paper in ethyl acetate–pyridine–water (8:2:1, by vol.) (solvent *A*).

The sugars were detected and estimated by the aniline hydrogen phthalate method of Wilson (1959) and results were calculated as percentage of each glycosyl radical present. Standard solutions of the monosaccharides were made up in saturated benzoic acid solution and stored at 4°. Definite volumes of standard and unknown solutions were applied to chromatograms by means of E-mil pipettes (H. J. Elliott Ltd., Treforest, Glamorgan).

Uronic acids were separated by chromatography with ethyl acetate–acetic acid–pyridine–water (5:1:5:3, by vol.) (solvent *B*) in a tank equilibrated with ethyl acetate–pyridine–water (40:11:6, by vol.) (Fischer & Dörfel, 1955).

Electrophoresis. Sugars were separated from amino acids on strip supports of Whatman no. 1 paper (57cm. × 25cm.; effective length 45cm.). The starting line was 19cm. from the anode. Electrodes were of platinum foil.

Electrophoresis was carried out with acetic acid (8%, v/v)–formic acid (2%, v/v) buffer, pH2·1, at 2000v and 50ma for 1hr. in a tank filled with white spirit (Esso Petroleum Co. Ltd., Cambridge), which was cooled by water in a glass coil.

Sugars were detected by the aniline hydrogen phthalate dip and the amino acids by a 0·2% solution of ninhydrin in acetone, which was used as a dip.

Radioactive chemicals. The glucose used was D-[6-^{3}H]-glucose of specific activity 1·3c/m-mole, D-[1-^{14}C]glucose of specific activity 4·0mc/m-mole (The Radiochemical Centre, Amersham, Bucks.) and D-[1-^{3}H]glucose of specific activity 350mc/m-mole (Volk Radiochemical Co., Skokie, Ill., U.S.A.).

Counting procedure. The chromatograms and electrophoretic supports were cut down their length into a series of consecutively numbered 1cm.-wide strips; each series corresponded to an initial spot on the starting line. The strips were placed in the counting vials together with 0·5ml. of toluene scintillator fluid [1,4 bis-(5-phenyloxazol-2-yl)benzene (0·005%) and 2,5-diphenyloxazole (0·35%) dissolved in toluene] and counted for 10min. in a scintillation counter (Tri-Carb Liquid Scintillator Spectrometer, series 314A; Packard Instrument Co. Inc., La Grange, Ill., U.S.A.). The position of the radioactive material found in the strips was compared directly with marker spots that were shown up by the aniline hydrogen phthalate dip on the same chromatogram or electrophoretogram.

Radioautography. (*a*) Light microscopy. The tissue was fixed with glutaraldehyde (6%, v/v) in phosphate buffer, pH7, for 2hr. It was then washed, dehydrated in ethanol and embedded in paraffin wax (m.p. 49°). Sections (6–8μ) were collected on gelatine-coated glass slides. After removal of the wax in xylene the sections were hydrated in an ethanol series and placed finally in water or in EDTA (50mM in phosphate buffer, pH7). The EDTA extraction was carried out at 70° for 2–5hr. and the extraction with water was carried out at room temperature.

After extraction the slides were coated with photographic emulsion (Ilford L–4 diluted 1:1 with water) by pipetting the solution on to the slides and allowing them to drain. This latter operation was done in the dark room with a safety light (Wratten series 1; 25w bulb). The coated sections were stored for 3 days in light-tight boxes containing silica gel and then developed and fixed (Ilford ID–19 for 6min., Kodafix diluted 1:5 for 10min.). After washing the slides were dried and examined with phase optics.

(*b*) Electron microscopy. The tissue was fixed in $KMnO_4$ (4%, w/v) containing NaCl (90mM), KCl (3·5mM) and $CaCl_2$ (1·2mM) (Pickett-Heaps & Northcote, 1965) for 2hr. After washing in water it was dehydrated in ethanol series and finally in propylene oxide and then embedded in Araldite resin.

Sections were cut on a mechanical advance microtome with glass knives. 'Gold' and 'silver' sections were collected on carbon-coated copper grids and these were stained with lead (Millonig, 1961) for 5–15min. After staining the sections were coated with carbon (Koehler, Mühlethaler & Frey-Wyssling, 1963) and then photographic emulsion (Ilford L–4) according to the method of Caro & van Tubergen (1962). The grids after being coated with the photographic film were kept for various periods (2–4 weeks) at 4° in small light-tight boxes containing silica gel.

The film was developed in filtered Ilford ID–19 for 1min., washed in water and fixed in Kodafix (diluted 1:4) for 1min. The grids were then washed and soaked in water for 10min., dried and examined with an electron microscope (Phillips EM 100) at 80kv.

RESULTS

Radioautographic observations

The sections of the tissue used for the radioautographic work were very similar to those taken from the fresh tissue without the incubation in the radioactive glucose (Mollenhauer, Whaley & Leech, 1961) and thus there seems to be little damage or alteration of the metabolism of the root tip either from the incubation procedure up to a period of 3hr. or because of the incorporated radioactivity.

Continuous incorporation. Four root tips were used for each experiment. The tips (1·5mm. long) were excised with a clean razor blade on a glass slide and immediately placed into a solution of D-[6-^{3}H]glucose (0·2ml., 0·2mc) in a small test tube lightly plugged with cotton wool. After incubation for 0·5–6·0hr. at room temperature the tips were removed, washed in water and prepared for electron microscopy. Similar experiments were performed with D-[1-^{3}H]glucose (0·1ml., 0·1mc) with incubation times of 0·5–4·0hr.

After exposure of the root tips to the radioactive glucose for 30min. labelled material was found in the golgi apparatus, its associated vesicles, the wall and in the new material of the wall under the plasmalemma (*m* and *n* on Plate 4). Similar results were also observed after exposure for 15min. to the D-[6-^{3}H]glucose and during the next few hours the label accumulated steadily in the external layer of material surrounding this tissue. This layer is presumably the protective slime of the root-cap cells (*k* and *l* on Plate 3).

The golgi bodies of the internal root-cap cells are not hypertrophied (Mollenhauer, 1959) and the vesicles incorporated into the wall of these cells are smaller than those of the outer root cap (Pickett-Heaps & Northcote, 1965). The radioactive label is found in the small golgi vesicles and in positions at the wall where it is known that vesicular incorporation occurs (*p* on Plate 4), but it is also found in various other parts of these cells. A continuous incorporation of labelled material into the wall was evident when the period of incubation of the tissue in the labelled glucose was extended for several hours.

An important difference in the labelling pattern of the organelles of the outer and more internal cells of the root cap can be seen by comparison of the plastids in these cells (*s* and *t* on Plate 5). This difference indicates variations in the metabolism of the cells. The outer root-cap cells of excised tissue that was incubated in media containing only the very small amounts of the D-[6-^{3}H]glucose (47μg.) contained plastids in which only very little of the label had been incorporated into their starch grains (*t* on Plate 5). In the more internally situated cells of the same tissue the plastids contained starch that had become appreciably labelled (*s* on Plate 5). Plastids in these two types of cell in a single radioautographic section are shown in (*s*) and (*t*) on Plate 5.

Long-pulse incorporation. The previous experiments showed that the labelled glucose was incorporated very quickly into the material of the cell, and an examination of the sections of the excised roots indicated that these underwent cytological changes after about 3hr. Pulse experiments with intact tissue were therefore designed to investigate the metabolism of the supplied radioactive glucose in more detail.

Intact seedlings were gently washed and blotted dry with filter paper. These were then placed with one of their root tips dipping into a drop (0·05ml.) of D-[6-^{3}H]glucose solution (2mc/ml.) placed on a clean glass slide covered by a Petri dish. The atmosphere within the Petri dish was kept moist by means of damp filter paper that was not in contact with the seedlings. Seedlings were removed and prepared for electron microscopy after 15 and 30min. incubation. The remainder of the plants were incubated for 45min. in the radioactive solution and were then washed with water, and the particular root tip was placed in a solution of unlabelled D-glucose (1·0ml., 500μg.) (i.e. an unlabelled glucose 'chase') in a Petri dish for 0·25–3·25hr. and then prepared for electron microscopy.

A radioautograph of root-cap cells after incubation in a solution of unlabelled glucose for 2·25hr. is shown in (*q*) on Plate 5. The label is no longer present to any extent in the golgi apparatus nor in the associated vesicles. The label is much more randomly distributed in the cell but there is a considerable concentration of labelled material in the starch grains of the plastid and in the slime and walls (*q* and *r* on Plate 5).

The internal root-cap cells also show a similar random distribution of the label after the chase with unlabelled glucose although here again the accumulation of labelled material in the starch grains and wall is very apparent (*o* on Plate 4).

Short-pulse incorporation. The incubation procedure was similar to that described for the long-pulse incorporation experiments. The intact seedlings were treated with D-[6-^{3}H]glucose (0·05ml., 5mc/ml.) and were removed and prepared for electron microscopy after 5 and 10min. incubation. The remainder of the plants were placed in unlabelled glucose (1·0ml., 1·0mg.) for 10, 30 and 60min. before being prepared for electron microscopy.

The results of these experiments are shown in (*a*)–(*j*) on Plates 1–3. These radioautographs were prepared from sections all of which were exposed to the photographic emulsion for the same period (3 weeks).

After 5min. exposure to the radioactive labelled glucose, derivatives appear within the cell but these are confined mainly to those areas of the section representing the golgi apparatus of the cell (*b* on Plate 1). The labelling of the cytoplasm is generally sparse and no labelled material appears in the wall although new wall substance can be detected in the section under the plasmalemma (*a* and *b* on Plate 1). Within 10min. the golgi apparatus and its associated vesicles are markedly labelled (*c* on Plate 1) but even at this stage there is

little labelled material deposited in the wall (*d* on Plate 1). After 10min. incubation in the radioactive labelled glucose followed by incubation in unlabelled glucose for periods of 10, 30 and 60min., there is a progressive loss of radioactivity from the golgi apparatus and vesicles and a progressive increase in the radioactivity of the material of the wall and slime (*e–j* on Plates 2 and 3).

Radioautography with the light microscope. Excised root tips were placed in D-[6-^{3}H]glucose (0·1ml., 2mc/ml.) in a test tube for 2hr.

The sections from these root tips were extracted with EDTA or cold water before being prepared for microscopy. In the sections treated with water, radioactive labelled material was detected in the slime layer around the root cap and in the walls of the cells (*u* on Plate 6), whereas in the sections treated with EDTA the labelled material of the slime layer was nearly all removed (*v* on Plate 6).

Chemical investigation

Root tips (5·8mg.) were divided into cap (2·4mg.) and meristematic portions (3·4mg.) and each portion was separated into α-cellulose and hemicellulose fractions. These were analysed for their component sugars and the results are shown in Table 1.

The course of the incorporation of the radioactive glucose into the root tips was followed by the analysis of material obtained from the tips after incubation in the glucose at room temperature for 0·25, 0·5, 1, 2, 3 and 4hr. For each incubation, six root tips cut from two seedlings were placed in a solution of D-[6-^{3}H]glucose (0·2ml., 1mc/ml.). After incubation, the tips were washed with water and placed in ethanol (50%, v/v). The root tips were subdivided into a cap (the apical 0·7–1·0mm. segment) and a meristematic portion (the remaining 0·5–0·7mm. segment) and these were analysed separately into α-cellulose and hemicellulose fractions, which were hydrolysed and the monosaccharides were separated chromatographically (solvent *A*). The results are shown in Tables 2 and 3.

The strips of filter paper carrying the origin spots of hemicellulose hydrolysates (Table 2) after the 1, 2, 3 and 4hr. incubations were collected, washed with toluene to remove the scintillator, dried in a current of cold air and eluted with water, and the aqueous solution was divided into two halves. One half was rehydrolysed with sulphuric acid (3%, w/v). The neutralized hydrolysate and the unhydrolysed solutions were freeze-dried. The residues were run on a chromatogram (solvent *B*) with galacturonic acid and glucuronic acid markers. No radioactive material coinciding with the uronic acid markers was detected. Two components (A and B) with positions relative to galacturonic acid (R_{GalA}) 0·85 and 1·4 respectively were found in the unhydrolysed material, and relatively small amounts of component A together with mannose and galactose were detected in the hydrolysed solution.

Separate experiments to determine the total amount of radioactivity taken up by the excised roots and the percentage recovered in the sugars of the hemicellulose, starch and α-cellulose fractions, the protein and lipid were carried out by incubating six root tips in the D-[6-^{3}H]glucose (0·2ml., 1mc/ml.) for 2hr. and washing the roots five times in unlabelled glucose (1ml., 2%, w/v) which was added to the remaining radioactive incubation

Table 1. *Composition of polysaccharide fractions of root tissue*

	% dry wt. of whole tissue	% dry wt. of segment	Sugar components of each polysaccharide (% of total sugars estimated)				
			Gal	Glc	Ara	Xyl	Man
Tip segment	41						
Hemicellulose fraction		10·5	15·1	42·5	25·5	17·5	Trace
α-Cellulose fraction		9·5	Trace	71·0	10·0	19·0	Trace
Meristem segment	59						
Hemicellulose fraction		14·0	13·0	32·0	27·0	28·0	Trace
α-Cellulose fraction		10·0	Trace	73·0	15·0	12·0	Trace

EXPLANATION OF PLATES 1–6

Abbreviations: W, Wall of cell; S, slime layer; G, golgi apparatus; GV, vesicles derived from the golgi apparatus; ER, endoplasmic reticulum; P, plastid; M, mitochondria; EC, epidermal cell of the root meristem; V, vacuole; N, nucleus; XC, external root-cap cell; IC, internal root-cap cell. (*a*)–(*j*) show the passage of labelled material formed from a short pulse of radioactive labelled glucose over a period of 70min. in the outer root-cap cells. All the radioautographs were treated in an identical manner.

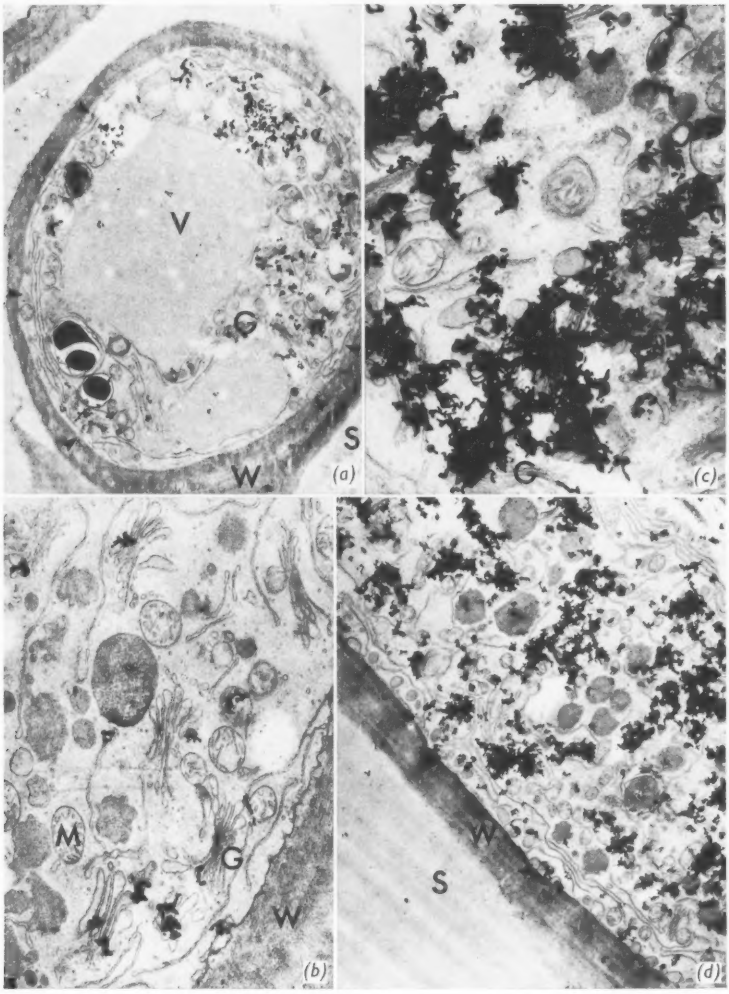

Plate 1. (*a*) Tissue exposed to D-[6-^{3}H]glucose (5mc/ml.) for 5min. before fixation; note the generally sparse labelling of the cell. New material being added to the wall is arrowed. Magnification × 3750. (*b*) A higher magnification of a cell from the same section as that shown in (*a*). Most of the labelled derivatives are located in the golgi apparatus. Magnification × 11 625. (*c*) Tissue had been exposed to D-[6-^{3}H]glucose (5mc/ml.) for 10min. before fixation. A massive accumulation of the labelled derivatives in the golgi apparatus is evident. Magnification × 14 435. (*d*) A longitudinal section of a root-cap cell, equivalent to that shown in (*c*). The labelled material is concentrated in the golgi apparatus. New material (arrowed) is being added to the wall but this and the wall are almost free of the label. Magnification × 3750.

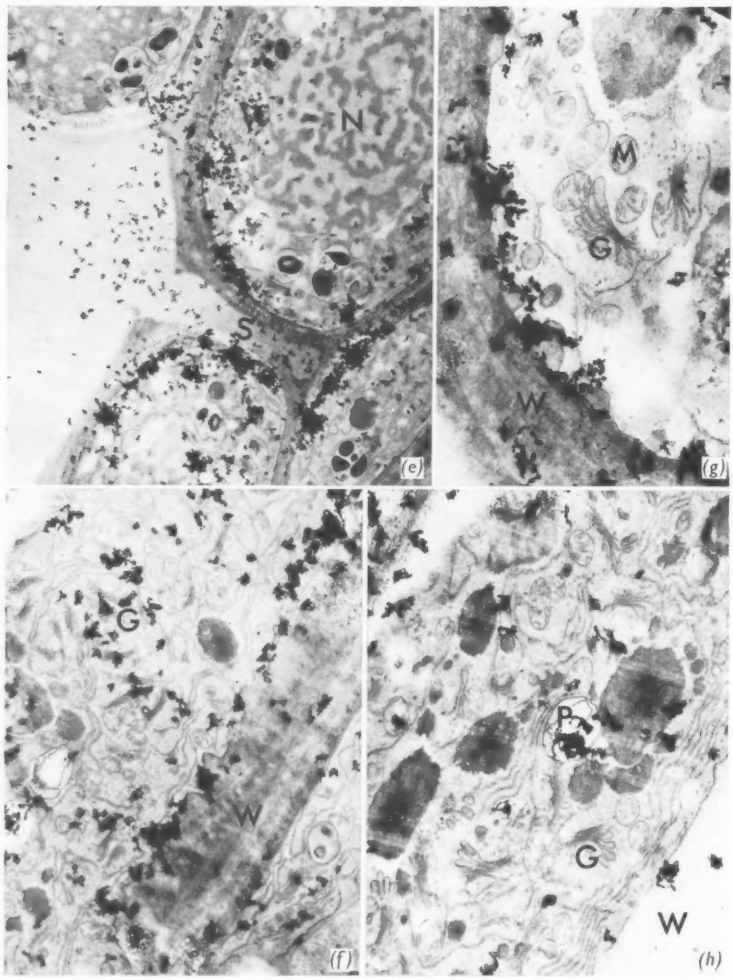

Plate 2. (*e*) After exposure to the labelled glucose (10 min.) the cells had been placed in a chase of unlabelled glucose (10 min.). Labelled material is apparent in many areas of the internal wall surfaces; traces of activity are also apparent in the slime material. The plastids show traces of radioactive incorporation. Magnification × 3000. (*f*) A higher magnification of cells equivalent to those shown in (*e*). The golgi bodies are still labelled, but now the new wall material also contains some activity. Magnification × 4875. (*g*) After exposure to the labelled glucose (10 min.) the cells had been placed in a chase of unlabelled glucose (30 min.). The golgi have lost almost all of their activity and the new wall material and the wall itself contain appreciable amounts of the labelled derivatives. Magnification × 10 685. (*h*) Another section equivalent to that shown in (*g*). The absence of labelled material in the golgi bodies is shown more clearly. The starch grain of the plastid has accumulated some radioactivity. Magnification × 7500.

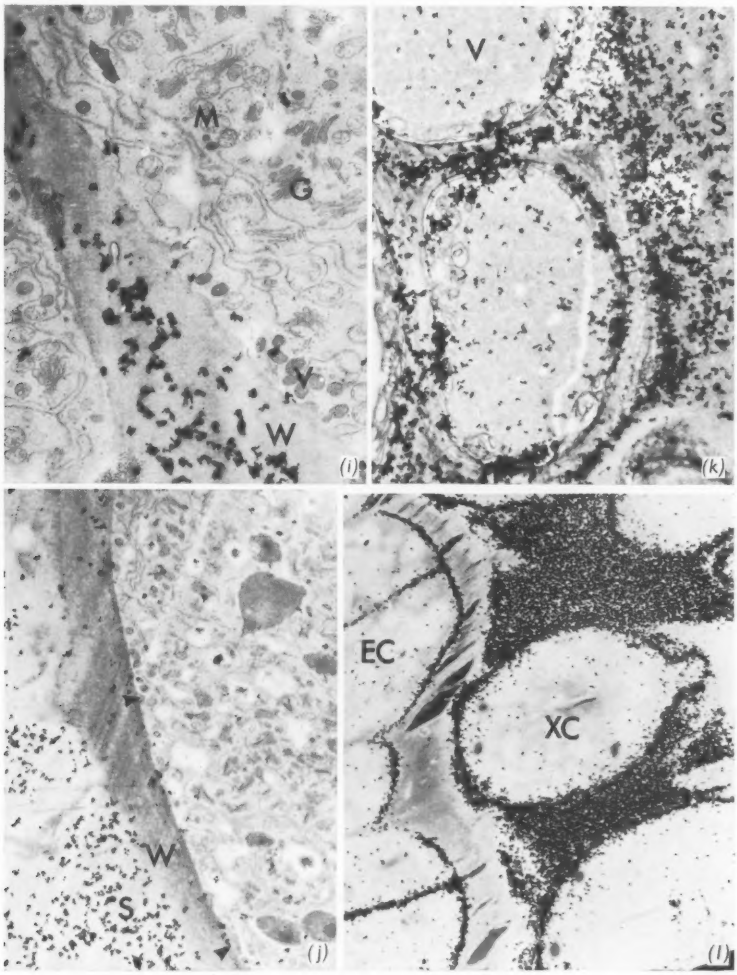

Plate 3. (*i*) The chase of unlabelled glucose in this experiment has been extended to 60 min. The cell contains little activity. The golgi vesicles contain very small amounts of label. There is a band of activity in the wall between the cells. Magnification × 5625. (*j*) A longitudinal section of a root-cap cell equivalent to that shown in (*h*). The small amounts of labelled material still present in the cytoplasm and the various cell organelles are clearly shown. An accumulation of radioactive material in the outer slime layers of the root cap is evident. Magnification × 3375. (*k*) Outer cap cell. Excised root tips were exposed to D-[1-^{3}H]glucose (1 mc/ml.) for 2 hr. The specific activity of this glucose was much lower than that of the D-[6-^{3}H]glucose. Large amounts of labelled derivatives can be seen on the internal surface of the wall, in between the cells, and particularly in the outer slime layer. Magnification × 3185. (*l*) Excised root tips were exposed to D-[6-^{3}H]glucose (1·0 mc/ml.) for 4 hr. An unstained transverse section of cap cells over the epidermal cells (EC) of the root meristem is shown. The large accumulation of labelled material in the slime layer of the cap cells is typical of that seen in all radioautographs taken from tips that were incubated for various periods in the labelled glucose without subsequent incubation in unlabelled glucose. Incorporation has also occurred in the walls of the epidermal cells. Magnification × 1910.

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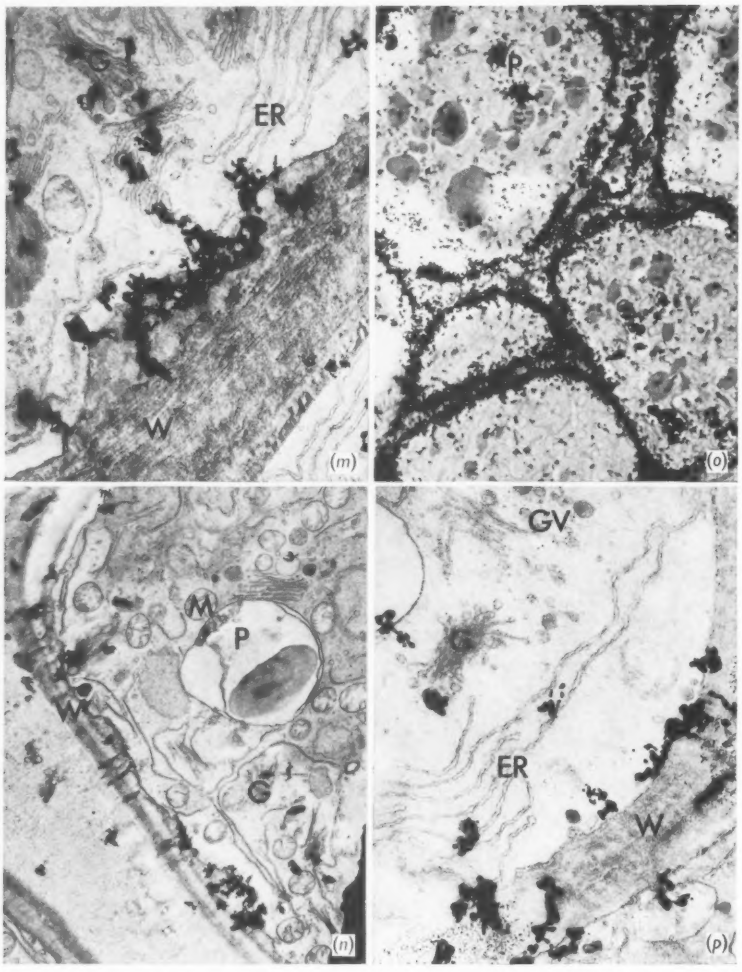

Plate 4. (*m*) Outer cap cell. The excised tissue was exposed to D-[6-^{3}H]glucose for 30 min. The radioautograph clearly shows the labelling of the new wall material and the golgi apparatus. Magnification × 13685. (*n*) Outer cap cell. The excised tissue was exposed to D-[1-^{3}H]glucose for 30 min. This labelled precursor was incorporated into the new wall material and golgi apparatus of the cell in a similar manner to that shown with D-[6-^{3}H]glucose (*m*). Magnification × 13685. (*o*) Long-pulse experiment. The intact root tips were exposed to D-[6-^{3}H]glucose (2 mc/ml.) for 45 min. followed by a chase of unlabelled glucose for 3·25 hr. The label is randomly distributed in these internal root-cap cells. The wall and starch grains show some accumulation of the label. Magnification × 1875. (*p*) Short-pulse experiment in which the experimental conditions were similar to those stated in the legend to (*g*). The golgi vesicles and the wall between these internal root-cap cells contain labelled material. Magnification × 16875.

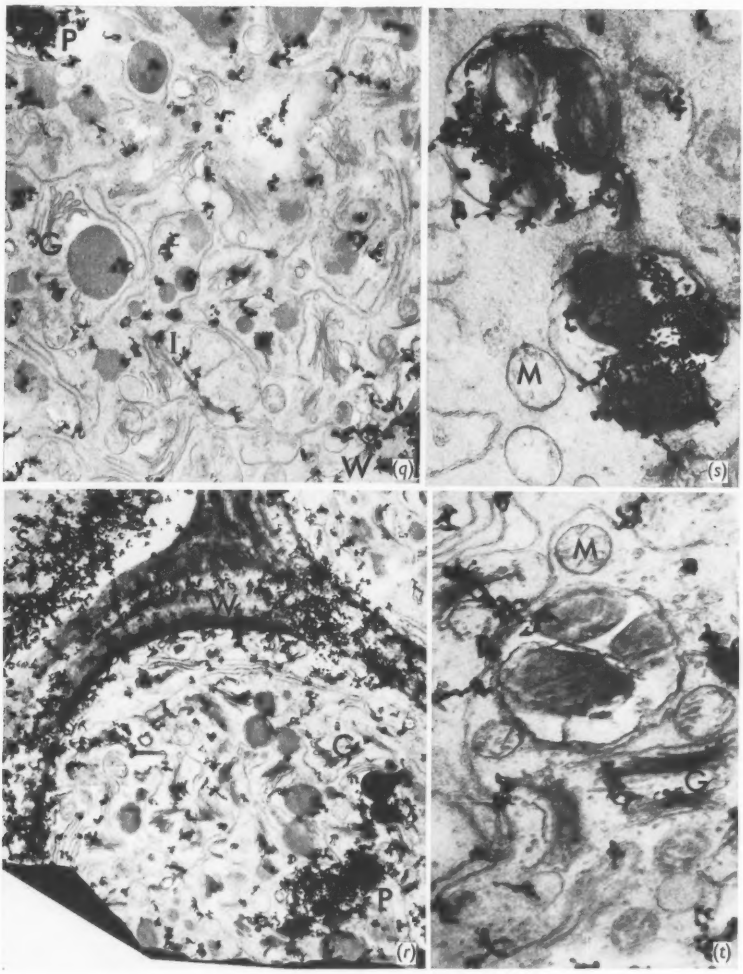

Plate 5. (*q*) Long-pulse experiment: outer cap cells. Intact root tips were exposed to D-[6-^{3}H]glucose (2 mc/ml.) for 45 min. followed by an incubation in unlabelled glucose for 2·25 hr. The label is randomly distributed in the cells. The golgi apparatus is free of activity. The starch in the plastid and the wall material is labelled. Magnification × 8810. (*r*) Long-pulse experiment: outer cap cell. Intact root tips were exposed to D-[6-^{3}H]-glucose (2 mc/ml.) for 45 min., followed by an incubation in unlabelled glucose for 3·25 hr. The starch in the plastids of the external cap cells is clearly labelled (compare with *t*). The golgi bodies are almost free of the label, but an incorporation of radioactivity into the wall is evident. The outer slime layer of the cells show an accumulation of labelled material. Magnification × 6975. (*s*) Excised root tips were exposed to D-[6-^{3}H]glucose (1 mc/ml.) for 1 hr. The starch in the plastids found in the internal root-cap cells is heavily labelled. Magnification × 15 750. (*t*) This photograph shows a typical plastid found in external root-cap cells after exposure of root tips to D-[6-^{3}H]glucose (1 mc/ml.) for 1 hr. It was taken from the same grid square as that containing the cell shown in (*s*). Magnification × 15 750.

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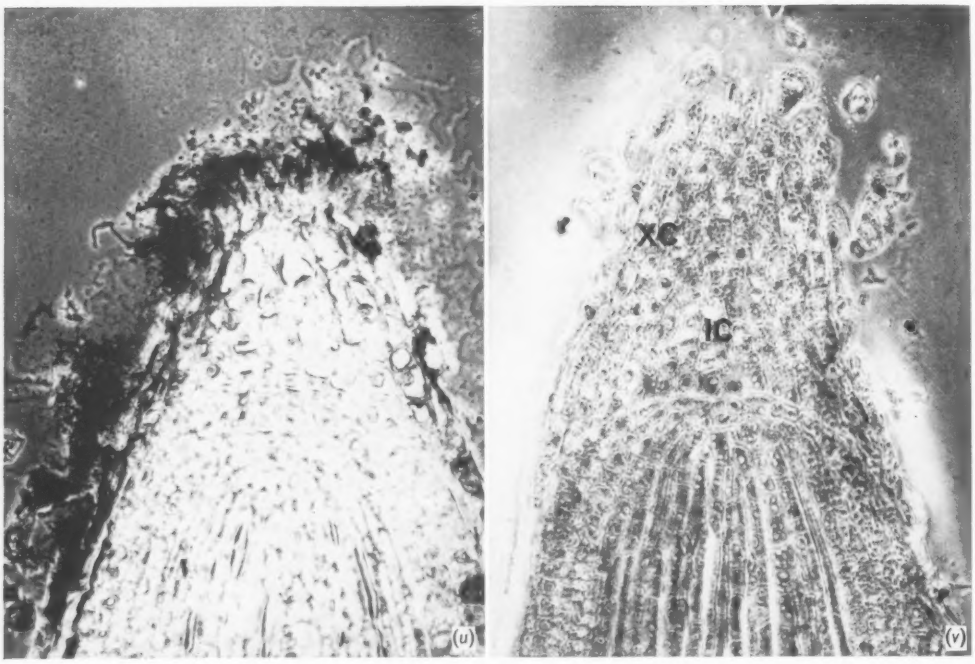


Plate 6. (*u*) Radioautograph of a longitudinal section of an excised root tip incubated in D-[6-^{3}H]glucose (0·1ml., 2mc/ml.) for 2hr. The section was extracted with water before the photographic film was put over the section. The labelled material of the slime layer of the cap is clearly visible. Magnification × 180. (*v*) Radioautograph of the same root tip used in the preparation shown in (*u*) but the section was treated with hot EDTA before the application of the photographic film. Magnification × 180.

Table 2. *Radioactivity of separated sugars isolated on chromatograms from the hydrolysate of polysaccharide fractions obtained from the cap segments of the root tips*

The root tips were incubated in D-[6-^{3}H]glucose (0·2 ml., 1 mc/ml.).

	Time of incubation (hr.)	Radioactivity					
		0·25	0·5	1	2	3	4
Hemicellulose fraction							
Total counts/min.		1805	2435	8330	11020	14810	14310
Origin material (% of total counts/min.)		9·8	10	9·1	9·8	13	11
Galactose (% of total counts/min.)		70	52	53	54	52	52
Glucose (% of total counts/min.)		11	21	22	17	14	15
Mannose (% of total counts/min.)		3·5	2·7	2·7	2·9	2·7	2·4
Arabinose (% of total counts/min.)		4·4	8·4	7·4	10	11	12
Xylose (% of total counts/min.)		1·4	5·4	5·5	7·5	8·1	8·2
Cellulose fraction							
Total counts/min.		958	6312	2930	10970	14800	12000
Origin material (% of total counts/min.)		5·5	9·5	5·5	3·2		
Galactose (% of total counts/min.)		85	65	61	61		
Glucose (% of total counts/min.)		8	18·5	29	34		
Mannose (% of total counts/min.)		1·6	3·8	2·0	1·3		
Arabinose (% of total counts/min.)		1	2·7	1·7	0·8		
Xylose (% of total counts/min.)		0·5	0·6	1·0	0·2		

Table 3. *Radioactivity of separated sugars isolated on chromatograms from the hydrolysate of polysaccharide fractions obtained from the meristematic segments of the root tips*

The root tips were incubated in D-[6-^{3}H]glucose (0·2 ml., 1 mc/ml.).

	Time of incubation (hr.)	Radioactivity					
		0·25	0·5	1	2	3	4
Hemicellulose fraction							
Total counts/min.		598	428	3410	18880	10840	12740
Origin material (% of total counts/min.)		11	21	19	13	22	13
Galactose (% of total counts/min.)		35	30	34	33	30	41
Glucose (% of total counts/min.)		30	34	22	27	20	14
Mannose (% of total counts/min.)		1·6	—	2·8	2·4	2·4	2·1
Arabinose (% of total counts/min.)		8	6·8	14	13	15	16
Xylose (% of total counts/min.)		14	8·4	7·5	10	9·3	14
Cellulose fraction							
Total counts/min.		1720	4146	15350	26100	30400	20130
Origin material (% of total counts/min.)		1	1	2	2·4	2·7	3·6
Galactose (% of total counts/min.)		24	29	46	55	58	49
Glucose (% of total counts/min.)		72	68	50	39	34	41
Mannose (% of total counts/min.)		1	1	0·6	1	0·5	1
Arabinose (% of total counts/min.)		1	0·6	0·2	1	0·7	2·3
Xylose (% of total counts/min.)		0·6	0·3	0·7	1·3	1·2	3·1

solution. The root tips were then washed in 10 ml. of cold ethanol (50%, v/v). The radioactivity of the solution before and after incubation and that of the ethanolic solution was measured so that the amount of radioactivity retained by the tissue could be calculated. The polysaccharide fractions were isolated from the roots in the usual manner, and these were hydrolysed and the sugars separated from the other material in the neutralized hydrolysates by electrophoresis at pH 2·1, and their radioactivity was measured. The radioactivity of the sugars in the polysaccharides represented 2·2% of the total radioactivity removed from the incubation solution by the tissue during the 2 hr. period, and of this recovered radioactivity 73% was present in the α-cellulose fraction, 23% in the hemicellulose fraction and 4% in the starch fraction.

The lipid of six root tips incubated with the D-[6-^{3}H]glucose (0·2 ml., 1 mc/ml.) for 2 hr. was extracted with methanol and diethyl ether. The

methanol extract contained 0·3% of the total radioactivity removed from the incubation mixture and the ether extract was completely unlabelled. The total radioactivity of the amino acids recovered from an electrophoretogram of a hydrolysate of the lipid-extracted root tips represented 0·4% of the total radioactivity removed from the incubation mixture.

Six excised root tips were incubated for 2hr. in a solution of D-[1-^{14}C]glucose (0·25ml., 0·1mc/ml.) at room temperature and the results for the analysis of the cap portions of the root tips are shown in Table 4. The material representing the origin spot of the hemicellulose hydrolysate (Table 4) was recovered from the filter-paper strip in the manner described above and it was re-run on a chromatogram with solvent *B*. The only radioactive material that was detected on this latter chromatogram ran at the same position as that of the marker galacturonic acid.

Radioactive galactose and glucose were eluted with water from the chromatograms of the hydrolysates of polysaccharides that had been obtained from root tips incubated in D-[6-^{3}H]glucose. The aqueous solutions were freeze-dried and the residues were run electrophoretically at pH 2·1 with glucose, galactose and amino acid markers. A clear separation of neutral sugars and amino acid markers was obtained. The sugars were found at 2cm. and the amino acids were detected 8–25cm. from the starting line towards the cathode. Of the total radioactivity of the applied radioactive sugars eluted from the chromatograms 90% was recovered in the neutral sugar position. The bulk of the remaining 10% formed a streak lying between the sugar and the beginning of the amino acid zones. An electrophoretogram of a fresh solution of the original D-[6-^{3}H]glucose used in the incubation experiments gave a similar quantitative pattern of the distribution of radioactivity. The sugars of the hydrolysates of the α-cellulose and hemicellulose fractions prepared from root tips incubated for 2hr. with D-[6-^{3}H]glucose were isolated from an electrophoretogram by elution with water of the zone found at 2cm. from the starting line. The total radioactivity of this zone was measured for each polysaccharide fraction and the eluates were freeze-dried and run on a chromatogram with solvent *A*. The distribution of radioactivity in the separated sugars was similar to that shown for the 2hr. incubation experiment in Table 2. The total amount of radioactivity in the various sugars accounted for 90% of the activity of the sugar zone of the electrophoretogram.

Table 4. *Radioactivity of separate sugars isolated on chromatograms from the hydrolysate of polysaccharide fractions obtained from the cap segment of the root tips*

The root tips were incubated in D-[1-^{14}C]glucose (0·25ml., 0·1mc/ml.) for 2hr.

	Radioactivity	
	Hemicellulose fraction	Cellulose fraction
Total counts/min.	1290	3260
Origin material (% of total counts/min.)	14·9	16·3
Galactose (% of total counts/min.)	16·9	36·4
Glucose (% of total counts/min.)	8·9	22·2
Mannose (% of total counts/min.)	9·6	4·1
Arabinose (% of total counts/min.)	27·4	12·0
Xylose (% of total counts/min.)	22·2	8·9

DISCUSSION

Previous electron-microscopic studies of the root tip of maize and wheat have shown that the outer root-cap cells possess characteristic hypertrophied golgi bodies (Branton & Moor, 1964; Mollenhauer & Whaley, 1963; Mollenhauer *et al.* 1961; Pickett-Heaps & Northcote, 1965). These organelles produce vesicles that seem to give rise to the massive amount of wall material and protective coating of the root tip. The cells of the tissue are thus particularly suited to a radioautographic study to characterize the material that the golgi produces and to provide direct evidence for the sequence of events leading to incorporation of material into the wall from these organelles.

The radioautography experiments have shown that within 5min. the label could be detected in the root-cap cells and was almost entirely confined to the golgi apparatus. Because of the treatment of the section before its examination by the electron microscope it seems likely that the radioactive material was insoluble in water and was therefore of high molecular weight. Within 15min. high-molecular-weight non-diffusible polysaccharides carrying the label could be isolated from the cells both in the hemicellulose and α-cellulose fractions. Analysis of these polysaccharides after incorporation of D-[6-^{3}H]glucose showed that over 70% of their radioactivity could be accounted for by the activity of the galactose content. Behind the root cap, in the portion of the root tip that contained the meristematic tissue, radioactive material derived from the administered D-[6-^{3}H]glucose could be detected on the radioautograph within 15min. of its application. In this tissue there was relatively no localization of material in the golgi bodies although some incorporation of the label into the wall had occurred. The polysaccharides extracted from this tissue contained 30–40% of their total radioactivity as galactose and the remainder was mainly that of glucose.

During the 3hr. incubation of the root tip in D-[6-^{3}H]glucose there was in both polysaccharide fractions of the root-cap tissue an increase in the proportion of the radioactivity of the glucose to the total radioactivity of the polysaccharides for the first hour of the incubation. The percentage of the total radioactivity of the galactose fell over this period since the radioactivity of the other sugars had slowly increased. It seems therefore that the pool of precursors of the glucans became saturated with the introduced radioactive glucose more slowly than the pools of the precursors of the slime and wall polysaccharides containing galactose. This would be expected in the outer root-cap cells where the passage of glucose through the precursor pools giving rise to these latter polysaccharides appeared to be very rapid indeed. The galactose, even in the α-cellulose, contributed a high proportion to the total radioactivity of this fraction, although the actual amount of galactose present is exceedingly small (Table 1). This again demonstrates very clearly the quick transformation of labelled glucose into the galactose polysaccharides of the various fractions of the wall.

The course of the labelling in the meristematic tissue (Table 3) was different from the root-cap tissues in that the radioactivity of the galactose slowly increased or remained fairly constant over a 3hr. period and that of the glucose decreased. It is likely that this difference resulted from the special processes of the cap cells that form material for their walls and the slime in addition to the polysaccharides required in the normal growth of the wall.

The radioautographs show that over a 3hr. period a very large amount of the radioactive label accumulated in the walls of the root-cap cells, and in the slime external to them, and most of the radioactivity of the tissue at this stage was confined to these regions. The chemical investigation has shown that the bulk of the radioactivity of the cells after 2hr. incubation in the radioactive glucose is present as polysaccharides and only relatively small amounts of labelled lipid and protein are present in the tissue. The radioactive material localized in the radioautographs therefore represented the position in the root tips from which the labelled polysaccharides were isolated.

The radioautographs of the pulse experiments show very clearly the dynamic sequence of the formation of the slime and wall material of the outer root-cap cells. The radioactive label was rapidly passed to the golgi apparatus through the vesicles and to the wall (*a–j* on Plates 1–3). The chemical analysis of the radioactive labelled hemicellulose derived from incubation with D-[6-^{3}H]glucose (Table 2) and D-[1-^{14}C]glucose (Table 4) showed that a large proportion of this radioactivity was due to the arabinose and galactose components. This result would be obtained if the radioactive label was rapidly incorporated into the polysaccharides of the pectic substances.

The radioactive material formed in the slime layer of the root cap was soluble in solutions of EDTA, as are the pectic substances.

The arabinose can be formed from the glucose by two pathways. One of these would involve the pentose phosphate pathway, which would form pentose sugars in which the radioactive label was randomized. The other route would involve a decarboxylation of UDP-glucuronic acid formed from UDP-glucose and an epimerization of the resultant UDP-xylose (Hassid, Neufeld & Feingold, 1959). This latter series of reactions would remove the primary alcoholic group of the glucose but leave the hemiacetal group intact. The occurrence of these two metabolic pathways for the formation of pentoses from hexoses would account for the difference in the relative amount of radioactivity recovered in the arabinose monomers when the seedlings were incubated with either D-[6-^{3}H]-glucose or D-[1-^{14}C]glucose (Tables 2 and 4).

The results of the pulse experiment show that variations in the metabolism of glucose occur in the cells of the root tip. These can be seen by the different appearance of the radioautographs of the internal and external cap cells and of these latter cells in the presence and absence of a large amount of exogenous glucose, supplied for the chase of unlabelled glucose. The outer root-cap cells synthesized very little starch because most of the soluble labelled glucose pool within the cell was channelled into the active golgi bodies of these cells and passed through them to accumulate in the slime and wall material. In the internal cells both starch and small amounts of wall material were finally formed from the soluble radioactively labelled pool. In these cells the golgi bodies and vesicles were not hypertrophied, and although they contain small amounts of labelled material they were not competing for the pool of the labelled glucose to the same extent as those of the outer root-cap cells. However, when unlabelled glucose was supplied starch synthesis did become apparent from the pool of labelled glucose within the cells of the outer root cap, and in the presence of the excess amount of glucose the label accumulated in the storage polysaccharides (*r* on Plate 5).

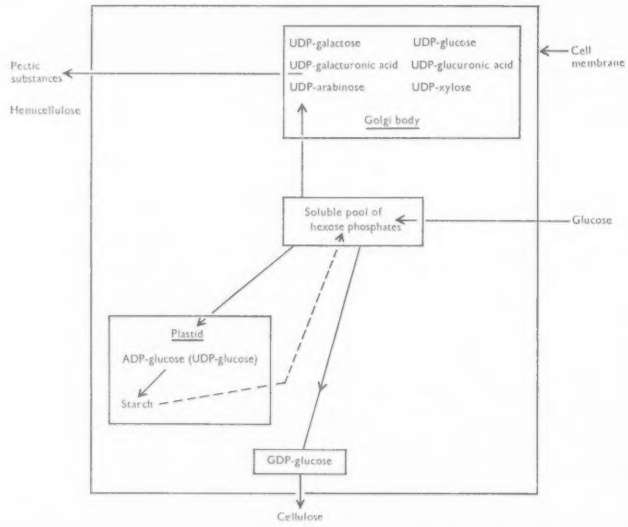
Our results are consistent with the idea that there occurs within the golgi apparatus a pool of precursors for the synthesis of polysaccharides that contain galactose, galacturonic acid and arabinose. These polysaccharides are probably identical with the pectic substances (Barrett & Northcote, 1965). The labelled glucose derivatives that enter the pool are not immediately available for cellulose

synthesis, and this is better regarded as arising from a separate pool of precursors that are derived from the same common pool of soluble glucose as those of the precursors of the galactose and arabinose (Andrews, Hough & Picken, 1965).

Galactose and arabinose monomers for polysaccharide synthesis are probably formed from UDP-galactose and UDP-arabinose that arise from the corresponding UDP-glucose compounds by epimerase activity; however, cellulose is probably formed from GDP-glucose (Barber, Elbein & Hassid, 1964) and starch from ADP-glucose and UDP-glucose (Leloir, Rongine de Fekete & Cardini, 1961; Recondo & Leloir, 1961). If this is correct then both the precursors and their locations within the cell are different for the different polysaccharides. These ideas are summarized in Scheme 1, which represents a working hypothesis for the routes of polysaccharide synthesis within the cell.

It has been indicated in animal (Caro, 1961; Caro & Palade, 1964; Porter, 1964; Wellings & Deome, 1961) and possibly in some plant tissues (Bonneville & Voeller, 1963) that the golgi apparatus is used to transport protein from the cell. The organelle may also take part in the formation and transport of mucopolysaccharides (Caro & van Tubergen, 1962; Godman & Lane, 1964; Fewer, Threadgold & Sheldon, 1964; Lane, Caro, Otero-Vilardebó & Godman, 1964; Peterson & Leblond, 1964). Manton and her colleagues (Manton, Oates & Parke, 1963; Manton & Parke, 1962; Manton, Rayns & Ettl, 1965) have shown that the golgi apparatus in certain flagellates can form inclusions of spicules and scales that are transported to the outer surface of the cell by means of vesicles formed from the cisternae of the golgi bodies, and excretory functions of the organelle in other plant tissues have been suggested (Bouck, 1962). During the growth of various plant cells material transported in golgi vesicles is incorporated into the enlarging wall (Gantt & Arnott, 1965; Rosen, Gawlik, Dashek & Seigesmund, 1963; Seivers, 1963) and also into the secondary thickenings of xylem elements and sieve tubes of sycamore stem and wheat seedling (Pickett-Heaps & Northcote, 1965; Wooding & Northcote, 1964, 1965). The polysaccharides laid down in the wall while the secondary growth takes place are derived from glucose, mannose, xylose and glucuronic acid, and very little pectic substance is used (Thornber & Northcote, 1961). Thus if polysaccharides are transported to the wall by the golgi apparatus at this secondary stage of development they are presumably those of the hemicellulose fraction.

The diverse nature of the materials transported by the golgi-body vesicles suggests that the function of the organelles varies with the metabolic conditions and state of development of the cell and that



Scheme 1. Scheme to show the separation of precursors and possible sites of synthesis of the polysaccharides formed by a plant cell.

generally it is concerned with the transport of materials in vesicles to sites outside the plasmalemma (Whaley, Kephart & Mollenhauer, 1959).

J. D. P-H. gratefully acknowledges the receipt of an Agricultural Research Council Postgraduate Studentship during the tenure of which this work was carried out.

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A Rat-Tissue Aminotransferase Acting on L-Tyrosine *O*-Sulphate

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(*Received* 8 *June* 1965)

1. Rat tissues have been shown to possess an aminotransferase that is active towards L-tyrosine *O*-sulphate and dependent on 2-oxoglutarate and pyridoxal phosphate. 2. Kidney, liver and pancreas have the greatest activity and the enzyme is localized mainly in the mitochondrial fraction in the liver and kidney cell. 3. The enzyme was shown to be distinct from L-tyrosine–2-oxoglutarate aminotransferase but its true identity was not established. 4. A procedure for the assay of the enzyme in crude tissue preparations was developed.

In previous studies from these Laboratories (Dodgson, Powell, Rose & Tudball, 1961; Powell, Rose & Dodgson, 1963) it was found that L-tyrosine *O*-sulphate, a peptide-bound component of some mammalian fibrinogens (Doolittle & Blombäck, 1964) and present in the free state in mammalian urines (Tallan, Bella, Stein & Moore, 1955), is rapidly converted into two major metabolites when injected intraperitoneally into the rat. These metabolites appear in the urine and have been identified as the sulphate esters of *p*-hydroxyphenylpyruvic acid and *p*-hydroxyphenylacetic acid respectively. The latter sulphate ester appeared to be the end product of L-tyrosine *O*-sulphate metabolism in these animals (Dodgson *et al.* 1961) and the former was considered to be an intermediate.

The present work concerns the transformation of L-tyrosine *O*-sulphate into *p*-hydroxyphenylpyruvic acid sulphate and establishes the existence in rat tissues of an aminotransferase that can accomplish the process. The enzyme appears to be distinct from L-tyrosine–2-oxoglutarate aminotransferase and L-phenylalanine–pyruvate aminotransferase.

EXPERIMENTAL

Materials and experimental animals. The ^{35}S-labelled and non-labelled sulphate esters of L-tyrosine and *p*-hydroxyphenylpyruvic acid were prepared as potassium salts by the methods described by Dodgson *et al.* (1961) and Rose & Powell (1963). Female M.R.C. hooded rats, aged 2–4 months, were used in all experiments.

Preliminary studies. In preliminary experiments aqueous suspensions (20%, w/v) of rat liver and kidney, prepared with the aid of a glass homogenizer, were incubated with L-tyrosine *O*[^{35}S]-sulphate under a variety of experimental conditions and in the presence of various added cofactors. Subsequently attempts were made to detect the formation of *p*-hydroxyphenylpyruvic acid [^{35}S]sulphate by subjecting samples of incubation mixtures to the paper-electrophoresis procedure described by Rose & Powell (1963). These experiments showed that *p*-hydroxyphenylpyruvic acid [^{35}S]sulphate was formed only if 2-oxoglutarate was present in the incubation mixture, suggesting that an aminotransferase enzyme was involved. By varying the concentration of L-tyrosine *O*[^{35}S]-sulphate and 2-oxoglutarate in the reaction mixture within the pH range 4–9, approximately optimum conditions for the enzyme were established qualitatively. These conditions were then used as a guide for the quantitative studies described below.

Estimation of enzyme activity. L-Tyrosine–2-oxoglutarate aminotransferase and L-phenylalanine–pyruvate aminotransferase (EC 2.6.1.5) can be assayed spectrophotometrically (Lin, Pitt, Civen & Knox, 1958). The method depends on the characteristic ultraviolet-absorption properties of the product obtained when boric acid is allowed to form a complex with the aromatic oxo acid formed as a result of transamination. *p*-Hydroxyphenylpyruvic acid sulphate also forms a boric acid complex with a characteristic absorption spectrum ($\lambda_{max.}$ 302mμ; Rose & Powell, 1963) and the possibility that this property might be employed to assay the enzyme acting on L-tyrosine *O*-sulphate was therefore examined. It soon became apparent that the procedure of Lin *et al.* (1958) could not be applied directly to the crude enzyme preparations under investigation. First, the high concentrations of protein interfered with the spectrophotometric measurement; secondly, even when this problem had been surmounted, recoveries of *p*-hydroxyphenylpyruvic acid sulphate were incomplete. A similar failure to obtain complete recovery of liberated oxo acid was experienced by Lin *et al.* (1958) in measuring the activity of L-tyrosine–2-oxoglutarate aminotransferase. This was traced to the presence of tissue oxo acid oxidase in the enzyme extracts and could be avoided by incorporating diethyldithiocarbamate (an inhibitor of these oxidases) in the reaction mixture. However, this material was ineffective in preventing the losses of *p*-hydroxyphenylpyruvic acid sulphate that occurred consistently with the crude enzyme preparation used in the present work.

An approximate measure of the losses was obtained by

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comparing the recoveries of added *p*-hydroxyphenylpyruvic acid sulphate from incubated (2hr. at 37°) reaction mixtures with those obtained for non-incubated mixtures. A loss of approx. 20% of the added ester occurred and this was independent of its concentration in the incubation mixture. Similar results were obtained with a number of tissues examined. In all determinations losses of *p*-hydroxyphenylpyruvic acid sulphate were assumed to be constant and were corrected for approximately by using a calibration curve with the assay procedure described below.

Assay procedure. The reaction mixture consisted of 0·3ml. of an aq. 10% (w/v) tissue suspension, 0·4ml. of 0·1M-phosphate buffer, pH7·3, 0·1ml. of aq. 0·22M-L-tyrosine *O*-sulphate and 0·1ml. of a solution containing 30μg. of pyridoxal phosphate and 6·0μmoles of 2-oxoglutarate in 0·1M-phosphate buffer, pH7·3. After incubation for 2hr. at 37° the reaction was stopped and protein precipitated by the addition of 0·2ml. of 30% (w/v) trichloroacetic acid. The mixture was cooled at 0° for 1hr. and precipitated protein was removed by centrifuging at 1500*g* for 15min. A 60μl. portion of the clear supernatant was mixed with 200μl. of 2·0M-sodium arsenate solution that contained boric acid (1·0M) and that had been adjusted to pH6·4 with HCl (test enol solution). A further 60μl. portion of the deproteinized incubation mixture was mixed with 200μl. of 2·0M-sodium arsenate that had been adjusted to pH6·4 with HCl (test keto solution). After standing for 20min. at room temperature, the extinction of the test enol solution at 302mμ was determined in 1cm. micro-cells (Hilger Uvispek), the test keto solution being placed in the blank cell. Control determinations were made in an identical manner except that L-tyrosine *O*-sulphate was added only after incubation and immediately before the addition of trichloroacetic acid. The spectrum of the product formed in the test determination was identical with that of pure *p*-hydroxyphenylpyruvic acid sulphate in the wavelength range 250–330mμ. An approximate measure of the amount of *p*-hydroxyphenylpyruvic acid sulphate liberated was obtained by subtracting the reading for the control determination from that for the test determination. The resultant value was then applied to the calibration curve constructed by incubating various amounts of the ester (range 0–200μg.) with a suspension of rat liver under the conditions of assay. One unit of activity is that amount of enzyme which liberates 1μg. of *p*-hydroxyphenylpyruvic acid sulphate under these conditions.

RESULTS

Optimum conditions for enzyme activity. The enzyme of rat tissues had optimum activity at pH7·3 and in the presence of a substrate concentration of 24mM (Figs. 1 and 2). The K_m for the reaction ($1{\cdot}68 \times 10^{-2}$M) was calculated by the method (case III) of Lineweaver & Burk (1934). The amount of *p*-hydroxyphenylpyruvic acid sulphate formed under the optimum conditions increased proportionately with the reaction time up to 2hr. at 37°.

The preliminary qualitative examination of rat liver for the enzyme showed that it was dependent on 2-oxoglutarate for activity. Maximum activity was obtained in the presence of 6mM-2-oxoglutarate

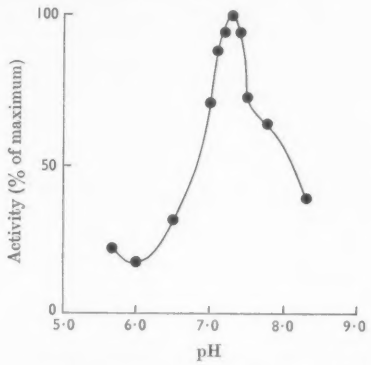

Fig. 1. pH–activity curve for the enzyme of rat-liver mitochondria acting on 22mM-L-tyrosine *O*-sulphate in the presence of 6mM-2-oxoglutaric acid, 68μM-pyridoxal phosphate and 0·1M-phosphate buffer.

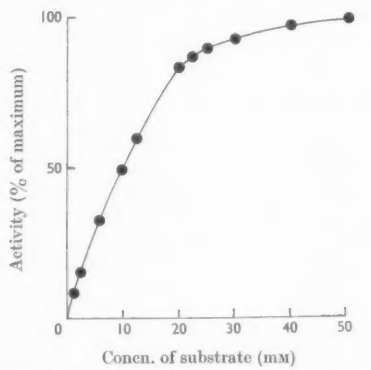

Fig. 2. Substrate concentration–activity curve for the enzyme of rat-liver mitochondria acting on L-tyrosine *O*-sulphate in the presence of 6mM-2-oxoglutaric acid and 68μM-pyridoxal phosphate. Incubation was carried out in 0·1M-phosphate buffer, pH7·3.

(Fig. 3). Glutamic acid was identified as a product of the reaction by paper chromatography as described by Lin *et al.* (1958).

Enzyme activity was detectable in crude tissue preparations without the need for prior addition of pyridoxal phosphate. Moreover, the addition of increasing amounts of pyridoxal phosphate to the reaction mixture did not markedly increase the enzyme activity of crude liver and kidney preparations. However, dialysis of liver preparations at 0° for 16hr. against 0·1M-phosphate buffer, pH7·3,

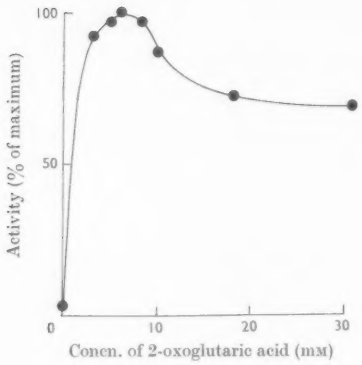

Fig. 3. Acceptor (2-oxoglutaric acid) concentration–activity curve for the enzyme of rat-liver mitochondria acting on 22 mM-L-tyrosine *O*-sulphate in the presence of 68 μM-pyridoxal phosphate. Incubation was carried out in 0·1 M-phosphate buffer, pH 7·3.

Table 1. *Distribution of* L-*tyrosine* O-*sulphate aminotransferase activity in rat tissues*

Details of the assay procedure are given in the text.

Tissue	No. of expts.	Activity (units/g. wet wt.) Mean	Range
Liver	6	3600	3000–3900
Kidney	4	4900	4100–5200
Pancreas	2	3270	2280–4260
Heart	2	1837	1804–1870
Lung	2	223	220–260
Brain	2	766	690–842
Spleen	2	333	300–366
Ovaries	2	500	420–580
Blood	2	165	152–178
Gut	2	478	400–556
Thyroid	2	652	640–664

completely inactivated the enzyme but activity could be restored by adding pyridoxal phosphate (5 μg.) to the reaction mixture. These results indicated that the enzyme was dependent on pyridoxal phosphate.

Distribution of enzyme activity in rat tissues. The amount of enzyme in various tissues of the rat is shown in Table 1. Suspensions of the tissues (10%, w/v) were prepared in 0·25 M-sucrose with the aid of a glass homogenizer. Whole organs were used, except for liver and intestine, where samples were taken. The intestine was opened and well washed with ice-cold water before homogenization. The results show that the enzyme is widely distributed in the rat. Kidney, pancreas and liver, however, appear to possess the greatest activity.

Intracellular localization of enzyme activity. The distribution of enzyme activity in the liver and kidney cell was compared with that of cytochrome oxidase (cytochrome *c*–oxygen oxidoreductase, EC 1.9.3.1), a typical liver mitochondrial enzyme, and lactate dehydrogenase (L-lactate–NAD oxidoreductase, EC 1.1.1.27), a supernatant enzyme. Cytochrome oxidase was determined by the method of Cooperstein & Lazarow (1951) and lactate dehydrogenase by the method of Delbruck, Schimmassek, Bartsch & Bücher (1959). Tissue fractionation was carried out in iso-osmotic (0·25 M) sucrose solution according to the scheme described by de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955). Preliminary experiments showed that sucrose did not interfere with the assay method. Uncontaminated cell nuclei were obtained by method 1 of Roodyn (1956).

The results (Table 2*a*) show that the bulk of the enzyme activity is associated with the mitochondrial fraction of both the liver and kidney cell. The non-particulate fraction of the kidney cell also possesses appreciable activity. This was shown to represent a true distribution of the enzyme by preparing a suspension of kidney in 0·25 M-sucrose with the aid of the Dounce-type homogenizer (see Allfrey, 1959), centrifuging for 30 min. at 100000*g* and measuring the distribution of activity between the debris and supernatant fractions obtained. By this treatment, artifacts that may have been introduced previously by rupturing the cell particles during homogenization and by the subsequent washing procedure were minimized. The results (Table 2*b*) indicate that the enzyme has a bimodal distribution in the kidney cell.

Attempts to release the enzyme from liver mitochondria. Several recognized procedures used for disrupting mitochondria were studied in attempts to obtain the enzyme in a soluble form. The effect of the treatment on the stability and solubility of the enzyme was determined from assays on the whole mitochondrial suspension before and after treatment and on the clear supernatant obtained by centrifuging the treated suspension at 25000*g* for 30 min.

Alternate freezing (with acetone–solid carbon dioxide mixtures at $-40°$) and thawing (37°) of rat-liver mitochondria, isolated as described above and suspended in water, 0·15 M-potassium chloride or 0·25 M-sucrose, was ineffective in releasing the enzyme into solution and resulted in a 10–15% inactivation. Treatment of the mitochondria with ice-cold acetone, followed by extraction with water, was similarly of no value. Ultrasonic disintegration of mitochondria suspended in water or 0·15 M-potassium chloride by treatment at 18–20 kcyc./sec.

Table 2. *Distribution of* L-*tyrosine* O-*sulphate–2-oxoglutarate aminotransferase in the rat liver and kidney cell compared with the distribution of cytochrome oxidase and lactate dehydrogenase*

Details of the tissue fractionation procedure used and enzyme assays are given in the text. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Results are the averages of three experiments, with the ranges in parentheses.

		Activity (% of the whole suspension)			Protein (% of whole suspension)
		L-Tyrosine *O*-sulphate aminotransferase	Cytochrome oxidase	Lactate dehydrogenase	
(*a*) Liver	Nuclei	8 (5–15)	5 (4–6)	9 (8–9)	12·5
	Mitochondria	53 (45–57)	62 (52–80)	7 (6–8)	15·5
	Lysosomes	13 (7–18)	2 (8–18	4 (4–5)	15·5
	Microsomes	7 (2–14)	7 (4–9)	13 (2–21)	13·5
	Supernatant	25 (25–27)	1 (0–2)	62 (48–75)	40·5
	Recovery	109	86	95	97·5
Kidney	Nuclei	8 (4–11)	5 (3–7)	5 (3–8)	10·0
	Mitochondria	35 (21–45)	44 (27–52)	4 (0–9)	19·0
	Lysosomes	19 (10–31)	17 (6–30)	4 (1–5)	10·0
	Microsomes	3 (2–5)	11 (6–14)	6 (5–6)	19·0
	Supernatant	32 (18–44)	3 (3–4)	62 (56–75)	47·0
	Recovery	97	80	81	105·0
(*b*) Liver	Particulate	89 (87–90)	61 (55–66)	23 (19–26)	
	Non-particulate	15 (14–16)	4 (0–8)	63 (49–82)	
Kidney	Particulate	61 (59–63)	74 (67–80)	13 (6–17)	
	Non-particulate	36 (35–37)	3 (1–5)	66 (50–72)	

for 5 min. at 5° by using the MSE ultrasonic disintegrator released little or no activity into solution. When mitochondria were suspended in 0·25M-sucrose and similarly disintegrated, however, a 75% solubilization of the enzyme without loss of activity was obtained.

Attempts to distinguish the enzyme from L-*tyrosine–2-oxoglutarate aminotransferase.* Kenney (1962) and Litwack, Sears & Diamondstone (1963) have shown that L-tyrosine–2-oxoglutarate aminotransferase is localized in the cell sap of the liver cell. This would apparently distinguish the enzyme from the L-tyrosine *O*-sulphate–2-oxoglutarate aminotransferase, which is localized mainly in the mitochondrial fraction (see Table 2) of this cell.

The separate identity of the two enzymes was also confirmed by a study of the amounts of the enzymes in the livers of rats that were receiving intraperitoneal injections of L-tyrosine, L-tyrosine *O*-sulphate and hydrocortisone. The rats were killed 4 hr. after injection and the amounts of the two enzymes determined in the excised livers. The results (Table 3) showed that the amount of L-tyrosine–2-oxoglutarate aminotransferase was elevated approximately threefold in the hydrocortisone-treated animals, in agreement with the observations of Kenney & Flora (1961), whereas the amount of the other enzyme remained unchanged. Injection of L-tyrosine *O*-sulphate had no significant effect on the amount of either enzyme, whereas injection of L-tyrosine elevated the L-tyrosine–2-oxoglutarate-aminotransferase activity to a greater extent than that of L-tyrosine *O*-sulphate–2-oxoglutarate aminotransferase.

Table 3. *Effect of intraperitoneal injections of hydrocortisone,* L-*tyrosine and* L-*tyrosine* O-*sulphate on the* L-*tyrosine aminotransferase and* L-*tyrosine* O-*sulphate aminotransferase of rat liver*

Animals were injected with 1 ml. of 0·9% NaCl (control) or with 1 ml. of 0·9% NaCl containing 10 mg. of hydrocortisone, 100 mg. of L-tyrosine or 5 mg. of L-tyrosine *O*-sulphate/200 g. body wt. 4 hr. before enzyme assay. L-Tyrosine *O*-sulphate aminotransferase was assayed as described in the text. L-Tyrosine aminotransferase was assayed by a modified Briggs reaction as described by Litwack (1962). Results are the means ± S.E.M. of six experiments.

	Aminotransferase activity (units/g. wet wt.)	
	L-Tyrosine *O*-sulphate	L-Tyrosine
None	3130 ± 470	3·6 ± 0·4
0·9% NaCl	3330 ± 960	3·7 ± 0·7
Hydrocortisone	3370 ± 600	10·4 ± 0·3
L-Tyrosine	4960 ± 1020	7·5 ± 0·9
L-Tyrosine *O*-sulphate	3530 ± 590	4·2 ± 0·6

Attempts to distinguish the enzyme from L-*phenylalanine–pyruvate aminotransferase.* Although it seemed unlikely that L-phenylalanine–pyruvate aminotransferase could be identical with the aminotransferase acting on L-tyrosine *O*-sulphate, preparations of the former enzyme were obtained according to the procedure of Lin *et al.* (1958) and tested for activity towards the two compounds. The preparations exhibited considerable activity towards L-phenylalanine but were completely inactive towards L-tyrosine *O*-sulphate under a wide variety of experimental conditions.

DISCUSSION

The widespread distribution in rat tissues of an enzyme that deaminates L-tyrosine *O*-sulphate to produce *p*-hydroxyphenylpyruvic acid sulphate has been clearly shown. The enzyme is presumably responsible for the rapid and efficient deamination of L-tyrosine *O*-sulphate that occurs when the ester is injected intraperitoneally into the rat. Although the affinity for substrate is low ($K_m 1{\cdot}68 \times 10^{-2}$ M) the widespread distribution of the enzyme may account for the observed efficiency of the system *in vivo*. The enzyme might tentatively be called L-tyrosine *O*-sulphate–2-oxoglutarate aminotransferase, and its distinction from L-tyrosine–2-oxoglutarate aminotransferase and the L-phenylalanine–pyruvate aminotransferase described by Lin *et al.* (1958) seems certain. However, it has not been established whether the enzyme shows absolute specificity towards L-tyrosine *O*-sulphate. The enzyme has some characteristics in common with a 2-oxoglutarate-dependent aminotransferase already recognized (Nakano, Tsuchiya & Danowski, 1963) that deaminates di-iodo-L-tyrosine. Further, the tissue distribution of the two enzymes and their localization in the kidney cell are very similar. Nakano & Danowski (1964) have shown that rat-kidney mitochondria possess a number of enzymes that deaminate derivatives of L-tyrosine, and the identification of L-tyrosine *O*-sulphate aminotransferase as a separate enzyme must therefore await its purification.

This work was supported by the Royal Society and by U.S. Public Health Service Research Grant no. AM01982-07 from the Arthritis and Metabolic Diseases Division. T. H. F. and R. A. J. are grateful to the Medical Research Council and the Department of Scientific and Industrial Research respectively for studentships.

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Synthesis of Connective-Tissue Components

THE EFFECT OF RETINOL AND HYDROCORTISONE ON CULTURED LIMB-BONE RUDIMENTS

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(*Received* 9 *June* 1965)

1. The action of excess of retinol on chick limb-bone rudiments cultured in chemically defined media has been investigated. 2. After 6 days *in vitro*, the hexosamine and hydroxyproline contents of the retinol-treated rudiments were much less than those of their paired controls. 3. Synthesis of these compounds, however, was not correspondingly decreased. 4. The control rudiments released hexosamine- and hydroxyproline-containing materials into the culture medium; a greater proportion of the hexosamine and hydroxyproline synthesized was liberated from the retinol-treated than from the control rudiments. 5. Although hydrocortisone in physiological concentrations prevented excessive hydration of the rudiments in culture, it did not significantly inhibit the changes induced by excess of retinol.

Fell & Mellanby (1952) showed that the cartilaginous limb-bone rudiments from 7-day embryonic chicks became soft and rubbery when they were grown as organ cultures for 8 days in medium containing excess of vitamin A (10i.u./ml.). The treated rudiments were much smaller than their paired controls in normal medium, and on histological examination they were found to have lost most of the basophilic (metachromatic) material from the matrix. Subsequently Fell, Mellanby & Pelc (1956) observed by radioautography that one of the first effects of the vitamin on cartilage was to prevent the incorporation of inorganic sulphate ($^{35}SO_4^{2-}$) into the matrix; this was followed by a loss of sulphate from the matrix, which accompanied the disappearance of metachromasia in histological preparations.

In further experiments (Dingle, Lucy & Fell, 1961) it was shown that, after 6 days' cultivation in the presence of excess of vitamin A, the wet and dry weights and hexosamine content of the cartilaginous rudiments were only half those of paired controls grown in normal medium, whereas the amount of DNA was 80% of that in the controls. It was clear from this result that there had been a great decrease in the material of the matrix relative to the number of cells. Evidence was obtained that this loss of intercellular material was due to the increased release of an acid protease from the lysosomes of the cells (Lucy, Dingle & Fell, 1961; Dingle, 1961; Fell, Dingle & Webb, 1962), the enhanced release being compensated by a greater synthesis of the enzyme (Fell & Dingle, 1963).

Although the previous experiments demonstrated that excess of vitamin A destroys the organized structure of cartilage matrix, they shed no light on whether the vitamin also affects the synthetic activities concerned in the formation of intercellular substances. Chondrocytes might actively produce the low-molecular-weight components of matrix in the presence of excess of vitamin A, but be unable to form the characteristic macromolecular structures of the tissue owing to excessive proteolytic activity induced by the vitamin. We have investigated this possibility by estimating the total synthesis of two of the main constituents of the matrix, namely hexosamines and hydroxyproline, and by determining the extent to which these compounds are released into the medium. Interrelationships between the effects of vitamin A and hydrocortisone on the synthesis and release of hexosamines and hydroxyproline have also been studied, because it has been shown by Schryver (1965) that very small quantities of hydrocortisone are beneficial in maintaining organ cultures of cartilage in an apparently normal condition for 10 days.

MATERIAL AND METHODS

Organ culture

Explants. The humeri, femora and tibiae were removed from embryonic chicks at the 6–6½-day stage. Such rudiments consist of a continuous cartilaginous rod in which the three cellular zones characteristic of ossifying cartilage are clearly differentiated as small-celled epiphysial regions at either end, adjacent intermediate zones of proliferative

flattened cells merging with the area of hypertrophic cells that forms the middle segment of the shaft. The hypertrophic cartilage is covered by a two-layered periosteum, and the inner osteoblastic layer has deposited a thin osseous sheath on the surface of the cartilage. Embryonic chicks of the same age may vary in their degree of development, but the developmental stage of the limb-bone rudiments at explantation can be roughly assessed by the degree of calcification, as indicated by opacity to transmitted light, of the osseous collar. For this reason in each experiment the stage of calcification in the rudiments from the different chicks was recorded according to an arbitrary code, so that any variations in response due to developmental variations could be identified.

The explants included the cartilaginous 'model', the thin osseous sheath, the periosteum and a little adherent muscle; cartilage was by far the largest component.

In most of the experiments 'zero controls' were obtained by dissecting the rudiments from a number of embryos from the same batch of eggs as that used for the cultures; this material was not explanted but served as a basis of comparison with the explants.

Culture method. The culture vessels consisted of flat-bottomed glass dishes (3cm. diam. × 1cm. high). Each contained a square platform of stainless-steel mesh (Minimesh, F.D.P. quality, 1·5mm. × 0·010in. × 0·005in.; Expanded Metal Co. Ltd., West Hartlepool), the height of which was carefully adjusted so that the top was level with the surface of the medium. Two such vessels were enclosed in a single Petri dish (10cm. diam.) carpeted with a very thin layer of absorbent cotton wool saturated with sterile 0·9% NaCl solution. A large oval hole had been cut in the cotton wool to allow the culture vessels to rest on the glass floor of the Petri dish; this arrangement permitted transillumination of the rudiments when they were explanted or examined. The two culture vessels were separated by a silicone-treated glass rod whose length was about equal to the internal diameter of the Petri dish; the rod prevented any risk of fluid from the cotton wool 'creeping' between the adjacent walls of the vessels and thus contaminating the medium.

To compare the effects of two media, the two solutions were placed in a pair of culture vessels enclosed in the same Petri dish; the limb-bone rudiments from one side of an embryo were then explanted in one medium and those from the opposite side in the other. For most experiments 18 pairs of explants were grown.

The Petri dishes were stacked in small perforated-zinc and wire baskets, three to a basket, and placed in a small vacuum desiccator, which was then thoroughly gassed with 5% CO_2 in air and incubated at 38·5°.

Every 2 days the media were removed from the dishes by means of Pasteur pipettes, the tips of which were bent at an angle; a separate pipette was used for each dish. The used media were collected in Bijou bottles and stored in the deep-freeze.

Fresh medium was added to each culture vessel as soon as it had been emptied, the Petri dish was placed under the dissecting binocular microscope and the explants were turned over with the aid of glass-mounted needles with an angled shaft; the Petri dishes were closed, replaced in the desiccator, regassed and returned to the incubator.

At the end of the culture period (6 days) the explants and media were collected for examination.

Histology

In nearly all the experiments two sets of explants (i.e. three pairs of rudiments) were fixed as histological controls. They were placed for 30min. in 3% (v/v) acetic acid in Zenker's fluid followed by 1½–2hr. in Zenker's fluid without acetic acid. After thorough washing in many changes of tap water, dehydration and clearing in cedar-wood oil (three lots), they were embedded in paraffin wax and serially sectioned. Preparations were stained with toluidine blue [30min. in 0·5% (w/v) toluidine blue in 5% (v/v) ethanol] or with Delafield's haematoxylin and chromotrop.

Culture media

In Expt. 4 medium BGJ was used. This medium was developed by Biggers, Gwatkin & Judah (see Biggers, Gwatkin & Heyner, 1961). In its original form medium BGJ is hyperosmotic for chicken cells and therefore for all our experiments, except Expt. 4, the NaCl concentration was decreased to 530mg./100ml. and the KCl concentration to 40mg./100ml. It appears, however, both from our experiments and those made by J. J. Reynolds (unpublished work), that explants grown in an osmotically modified medium are histologically indistinguishable from those in the original medium BGJ. For all our experiments 5mg. of sodium acetate/100ml. was included in the medium, as this was found to give slightly improved results.

In previous studies the limb-bone rudiments were cultivated on a 'natural' medium containing plasma and embryo extract (cf. Dingle *et al.* 1961), the proteins of which presumably combined with the added retinol and thus prevented precipitation and decomposition of the vitamin. In the present experiments the use of natural media was impracticable as it contains large amounts of substances that would interfere with the estimation of hexosamine and hydroxyproline. Retinol is rapidly destroyed in saline solution containing dissolved O_2 (Lucy, 1965), and it cannot be recovered from medium BGJ after 24hr. at 37°; up to 60% of the vitamin may be recovered, however, when albumin, 1·0% (w/v), is added (Fig. 1). The presence of 0·5% of serum albumin allowed the recovery of about 45% of the added retinol after 24hr., so this concentration of protein was used in the culture experiments; higher concentrations of serum proteins often had a deleterious effect on the bone rudiments. Pig β-globulin (prepared in this Laboratory) and bovine γ-globulin (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex) were used in Expts. 4 and 3 respectively; in all the other experiments bovine plasma albumin, fraction V (Armour), was employed.

Synthetic retinol (Roche Products Ltd., London) was dissolved in ethanol and then added to the medium; the same quantity of ethanol (0·2%, v/v) was included in the control medium. Hydrocortisone sodium hemisuccinate (Solu-Cortef; Upjohn Co., Kalamazoo, Mich., U.S.A.) or Efcortalan (Glaxo Laboratories Ltd., Greenford, Middlesex) was dissolved in glass-distilled water and introduced into the medium, the same amount of water being put into the control medium.

Analytical methods

After being harvested, the rudiments were pooled in Tyrode solution, dried on filter paper and weighed. In some experiments the O_2 uptake of the explants was deter-

mined as described by Dingle *et al.* (1961). The bone rudiments were then dried over P_2O_5 *in vacuo* in the cold for 2 days and weighed again.

Subsequently the rudiments were hydrolysed in 5N-HCl for 4hr. at 100°. This procedure was used in all analyses; no attempt was made to determine the hydrolysis conditions for optimum yields of total hexosamines since the experiments were comparative and absolute quantities were not required. Samples of used culture media and samples of unused media as reagent blanks were dried *in vacuo* over P_2O_5 and then hydrolysed under similar conditions. After hydrolysis all samples were dried over P_2O_5 and KOH, then redissolved in water, and each sample was divided into two portions. One portion was analysed for total hexosamines by the method of Cessi & Piliego (1960), which was used with minor technical modifications. The presence of albumin in the medium did not affect the recovery of added glucosamine. In replicate analyses of culture media to which known quantities of hexosamine hydrochloride were added 99% of the hexosamine was recovered with an accuracy of ±4%. Analysis of 6-day (*ex ovo*) rudiments, in seven groups of 12 rudiments, gave a mean hexosamine content of 130μg./mg. dry wt., with a standard deviation of 24μg./mg. The second portion was evaporated to dryness and then hydrolysed for 24hr. at 110° in 6N-HCl; subsequently this hydrolysate was analysed for hydroxyproline by the procedure of Prockop & Udenfriend (1960). The accuracy of this estimation was similar to that of the hexosamine analysis. Glucose was measured by the method of Nelson (1944).

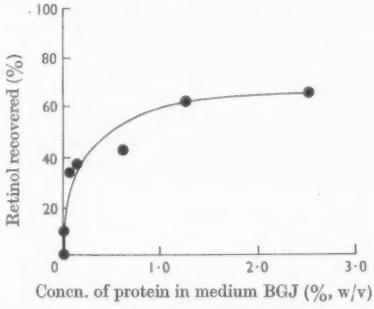


Fig. 1. Recovery of retinol from culture medium. Retinol (3μg./ml.) was added to medium BGJ containing different concentrations of bovine albumin. The medium was incubated for 24hr. at 37° and the vitamin extracted after the addition of 1 vol. of ethanol and 2 vol. of light petroleum (b.p. 40–60°). The extraction was repeated twice and, after evaporation under N_2, the retinol was assayed spectrophotometrically.

RESULTS

Biosynthesis of hexosamine and hydroxyproline. The use of synthetic culture media has made possible the estimation of materials liberated during the growth of chick limb-bone rudiments. Untreated rudiments were found to release more than 15μg. of hexosamine/rudiment after 6 days in culture; this amount was similar to that in the rudiment at the end of the experiment (Table 1). The rate of release was slightly greater towards the end of the culture period (Fig. 3). The liberation of hydroxyproline was also considerable and comparable with that contained in the rudiment (Fig. 2 and Table 1). These experiments demonstrate

Table 1. *Effect of retinol on the composition of cultured bone rudiments*

Humeri, femora and tibiae from 6-day embryonic chicks were grown in organ culture for 6 days on synthetic media containing protein. Eighteen pairs of rudiments were used in each experiment: one rudiment of each pair was grown in the presence of retinol. The rudiments in each group were pooled before weighing and analyses.

Expt. no.	Concn. of retinol (i.u./ml.)	Wet wt. (mg./bone)	Dry wt. (mg./bone)	Hexosamine (μg./bone)	Hydroxyproline (μg./bone)
1	3	1·77	0·193	15	1·1
	0	3·00	0·273	17	1·3
2	7	1·72	0·093	12	1·0
	0	3·71	0·271	15	1·9
3	10	2·52	0·166	10	1·2
	0	4·61	0·206	30	1·5
4	10	2·15	—	13	0·9
	0	3·95	—	26	1·9
5	10	0·71	0·075	5	1·7
	0	2·58	0·189	10	2·3
6-day (*ex ovo*)	—	0·58	0·062	8	0·5

that estimations of the synthesis of these compounds should take into account material released into the medium as well as that incorporated into the rudiment. The 'total synthesis' of any component is therefore defined in this paper as the increase in the material in the rudiment during the culture period plus the amount released into the medium at 2, 4 and 6 days.

Effect of vitamin A. At the lowest concentration of retinol used (3i.u./ml.; Expt. 1) there was little difference in appearance between the treated and control rudiments until the fourth day, when the former had become thinner and more flexible than the latter. By the sixth day they were much smaller than the controls, and metachromasia had nearly disappeared from the shafts. Staining remained fairly intense in the relatively large epiphyses and proliferative zones, however, where the matrix had a bloated water-logged appearance. The wet weight/dry weight ratio of the controls was 11·0 and that of the treated rudiments was 9·2. Growth in the presence of the vitamin was inhibited (controls, 0·27mg. dry wt./bone; treated rudiments, 0·19mg./bone). There was a slight increase (7%) in the rate of release of hexosamine into the medium of the treated rudiments as compared with their paired controls, but hydroxyproline release was unaltered. A slight decrease in the hexosamine and hydroxyproline content of the rudiments was observed at the end of the culture period (Table 1). The total synthesis of hexosamines was unaffected; total hydroxyproline synthesis was slightly depressed (Table 2).

In the presence of 7i.u. of retinol/ml. (Expt. 2) there was a distinct morphological effect by the second day, and at the end of the culture period the rudiments were shrunken, soft to the touch and very distorted. Metachromasia had disappeared from the shaft, and the matrix of the epiphyses had disintegrated into a loose web of metachromatic strands among which healthy-looking chondrocytes were entangled. Mitosis was surprisingly abundant throughout these explants. It was common even among the former hypertrophic cells, which, as described by Fell & Mellanby (1952), had completely lost their characteristic vacuolated structure and rounded form, and become amoeboid and basophilic. These cells proliferated to form small groups which completely filled many of the capsules that were originally occupied by single hypertrophic cells. The severity of these morphological effects was reflected in the big difference between the dry weights of the treated and control explants (Table 1). Analyses showed that in the treated rudiments the content of hydroxyproline was about half that of the controls but there was a smaller decrease in the amount of hexosamine; it is probable that in addition to these two substances other components of the tissue were also released and account for part of the difference in dry weight between the two sets of explants. It is noteworthy that the total synthesis of hexosamine and hydroxyproline was not diminished (Table 2), because although the treated rudiments contained less of these compounds than did the controls (Table 1) compensatory amounts were found in the culture medium.

The effect produced by 10i.u. of retinol/ml. depended on which protein was present in the medium. In Expt. 3 bovine γ-globulin was used and with this protein 10i.u. of retinol/ml. had a less severe action on the rudiments than was observed with either of the two other proteins employed. Though they were considerably smaller than the controls and very flabby, the treated rudiments were larger and less distorted than the corresponding explants in the other two experiments of this group (Expts. 4 and 5). The usual changes were well advanced in the shafts of the treated rudiments but the epiphysial cartilage still retained much of its metachromatic material. Total synthesis of hexosamine (Table 2) and binding of hexosamines by the rudiments (Table 1) were both severely diminished in Expt. 3. The hydroxyproline content of the treated rudiments was less affected than the hexosamine (Table 1), but considerably more hydroxyproline was released into the medium from the treated rudiments than from the controls (Fig. 2) and total synthesis of the amino acid was slightly greater (Table 2).

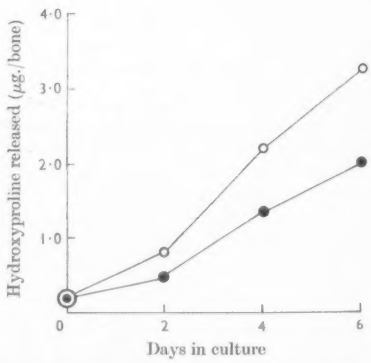
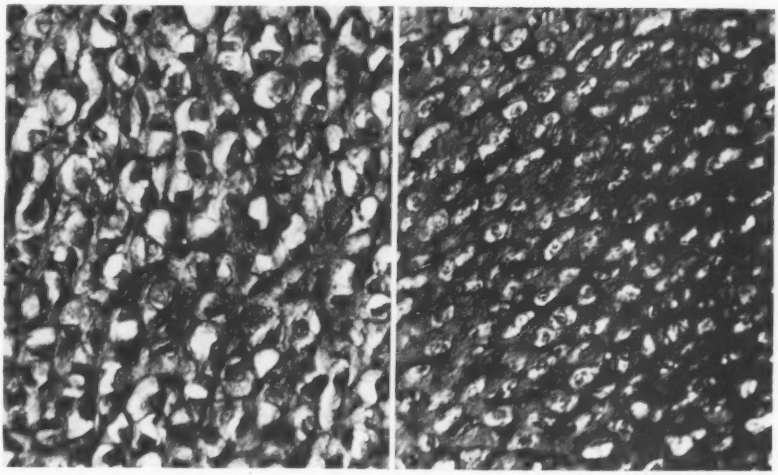
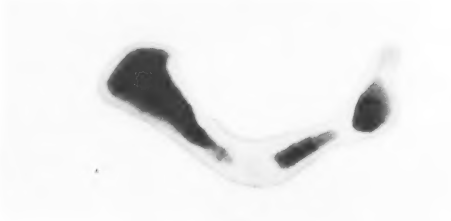


Fig. 2. Release of hydroxyproline by cultured bone rudiments. The conditions of culture were as described in Table 1. The media from 18 treated and 18 control rudiments were collected at each subculture and frozen until assayed for the hydroxyproline content. The results are expressed in μg. of hydroxyproline released/rudiment. ○, Retinol-treated (10i.u./ml.) cultures; ●, control.



(*a*) (*b*)

(*c*)



(*d*)

EXPLANATION OF PLATE 1

All sections were stained with toluidine blue. Photographs were by M. F. Applin. (*a*) Distal cartilage in explanted 6-day humerus grown for 6 days in hormone-free chemically defined medium (BGJ). Foamy matrix and enlarged cavities around many of the chondrocytes are present. Magnification × 440. (*b*) Similar region in humerus from the opposite side of the same chick used for (*a*), after 6 days' growth in medium BGJ containing hydrocortisone (0·1 μg./ml.). The matrix appears normal. Magnification × 440. (*c*) Control humerus grown for 6 days in the presence of hydrocortisone (0·1 μg./ml.). Magnification × 15. (*d*) Opposite humerus from the same embryo used for (*c*), after 6 days' cultivation in the presence of hydrocortisone (0·1 μg./ml.) and retinol (10i.u./ml.); the explant is smaller and there is extensive loss of metachromatic material. Magnification × 15.

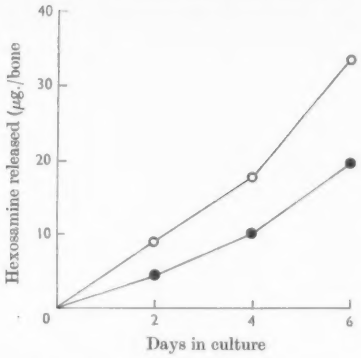


Fig. 3. Release of hexosamine by cultured bone rudiments. The conditions of culture were as described in Fig. 2. The results are expressed as μg. of hexosamine released/rudiment. ○, Retinol-treated (10i.u./ml.) cultures; ●, control.

Table 2. *Effect of retinol on the total synthesis of hexosamine and hydroxyproline by cultured bone rudiments*

Total synthesis represents the sum of material released into the medium and that incorporated into the rudiments. The experimental conditions were as described in Table 1.

Expt. no.	Concn. of retinol (i.u./ml.)	Hexosamine synthesized (μg./bone)	Hydroxyproline synthesized (μg./bone)
1	3	28	1·7
	0	28	2·0
2	7	23	3·7
	0	22	4·0
3	10	19	3·7
	0	39	3·0
4	10	37	—
	0	36	2·8
5	10	14	2·4
	0	29	3·7

Pig β-globulin was used in Expt. 4. The rudiments exposed to the vitamin were more affected than those in Expt. 3, being very much smaller than the controls and very soft; many had fractures in the zones of flattened cells (cf. Fell & Mellanby, 1952). The quantity of hexosamine bound by the treated rudiments (Table 1) was considerably less than in the controls, but the total synthesis was not inhibited (Table 2). During 6 days' cultivation the amount of hexosamine released into the medium from the treated rudiments was greater than that retained in the tissue (Fig. 3).

The most drastic changes were produced in Expt. 5, in which the medium contained bovine serum albumin. The treated explants rapidly shrank, became grossly distorted and by the sixth day were reduced to small gelatinous nodules; the effect of the vitamin was so severe in this experiment as to inhibit the synthesis of both hexosamine and hydroxyproline (Table 2).

Effect of hydrocortisone. Schryver (1965) has shown that the excessive hydration of cartilage rudiments that occurs during cultivation *in vitro* (Biggers, 1963) is prevented by the presence of small quantities of hydrocortisone. This observation has been confirmed in our experiments, and further investigations have been made to see if low concentrations of hydrocortisone can prevent hydration without interfering with the synthesis of hexosamines and hydroxyproline.

Excessive hydration of cartilage rudiments grown in medium BGJ containing added protein was reflected in the wet weight/dry weight ratio, which was high compared with that of rudiments examined directly *ex vivo* or after cultivation on natural medium. Thus the value of this ratio was 14·2 after cultivation on medium BGJ (a mean of 15 experiments each containing 15 rudiments, standard deviation 2·8), but only 8·8 for 6-day rudiments *ex vivo* (a mean of seven determinations each with 12 bones, standard deviation 1·02). A value of 10·3 after 6 days' cultivation on natural medium was observed in previous experiments (Dingle *et al.* 1961). Histological examination of rudiments grown on medium BGJ revealed that the cells in the deeper parts of the epiphyses and in the proliferative zone occupied large round cavities in a curious foamy matrix (Plate 1*a*).

Hydrocortisone was found to prevent many of these abnormalities. With 0·1 or 1·0 μg. of the hormone/ml. the matrix of the rudiments was much denser than in the controls (Plate 1*b*). The foamy appearance characteristic of the control explants was observed only near the articular surfaces and sometimes in limited patches in the zone of flattened cells; the chondrocytes were closely embedded in the matrix. The dry weight of the cultured rudiments was unaffected by concentrations of hydrocortisone below 1·0 μg./ml. but the wet weight was decreased by even the lowest concentration tested (Table 3). A mean value of 8·0 was obtained for the wet weight/dry weight ratio in five experiments (each with 15 rudiments) in which the concentration of hydrocortisone did not exceed 0·1 μg./ml.

With 1 μg. of hydrocortisone/ml. the hydroxyproline content of the rudiments was depressed, but with lower concentrations the hydroxyproline was increased (Table 3). This increase was due to

Table 3. *Effect of hydrocortisone on the composition of cultured bone rudiments*

The experimental conditions were as described in Table 1, except that one rudiment of each pair was cultivated in the presence of hydrocortisone.

Concn. of hydrocortisone (μg./ml.)	No. of rudiments	Wet wt. (mg./bone)	Dry wt. (mg./bone)	Hexosamine (μg./bone)	Hydroxyproline (μg./bone)
1·0	15	2·50	0·240	23	1·9
0·0	15	5·00	0·353	30	2·3
0·1	30	2·30	0·278	16	3·1
0·0	30	3·93	0·273	16	1·3
0·01	45	2·89	0·380	26	3·0
0·00	45	4·71	0·388	30	1·5
6-day (*ex ovo*)	48	0·75	0·088	9	0·6

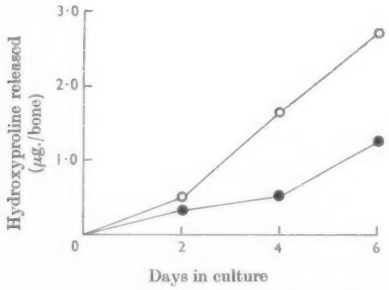


Fig. 4. Effect of hydrocortisone on the release of hydroxyproline by cultured bone rudiments. Eighteen paired rudiments were grown for 6 days on medium containing bovine serum albumin. One rudiment of each pair was grown on medium containing 0·1 μg. of hydrocortisone/ml. The hydroxyproline release was measured as described in Fig. 2. ○, Control; ●, hydrocortisone (0·1 μg./ml.) added.

Table 4. *Effect of hydrocortisone on the total synthesis of hexosamine and hydroxyproline by cultured bone rudiments*

Total synthesis represents the sum of material released into the medium and that incorporated into the rudiments. The experimental conditions were as described in Table 1, except that one rudiment of each pair was cultured in the presence of hydrocortisone.

Concn. of hydrocortisone (μg./ml.)	No. of rudiments	Hexosamine (μg./bone)	Hydroxyproline (μg./bone)
1·0	15	34	2·9
0·0	15	47	3·6
0·1	30	25	3·9
0·0	30	34	3·3
0·01	30	38	4·7
0·00	30	44	3·1

inhibition of the release of hydroxyproline into the culture medium (Fig. 4) combined with a small rise in the total synthesis of the imino acid (Table 4).

Release of hexosamines from the rudiments into the medium was also inhibited by hydrocortisone (Fig. 5), but this did not result in an increased quantity in the rudiments (Table 3) since total synthesis of hexosamines was depressed by the hormone at all the concentrations tested (Table 4). The depression of synthesis might perhaps be related to a small inhibition of glucose utilization (14%), and also to a slight inhibition of oxygen uptake, that were observed after the rudiments had been cultured for 6 days. In two experiments each on 15 pairs of rudiments, the mean rate of oxygen uptake without hydrocortisone was 1·6 μl./

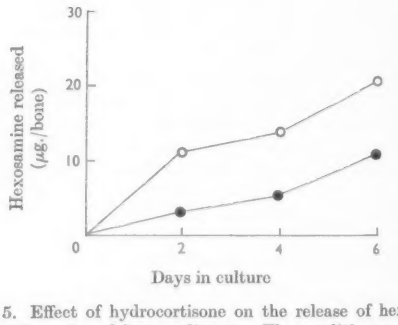


Fig. 5. Effect of hydrocortisone on the release of hexosamine by cultured bone rudiments. The conditions were as described in Fig. 4. ○, Control; ●, hydrocortisone (0·1 μg./ml.) added.

Table 5. *Effect of vitamin A on the synthesis of hexosamine and hydroxyproline by cultured bone rudiments in the presence of hydrocortisone*

The experimental conditions were as described in Table 1, except that both the control and experimental media contained hydrocortisone.

Expt. no.	Concn. of retinol (i.u./ml.)	Concn. of hydrocortisone (μg./ml.)	Hexosamine (μg./bone)		Hydroxyproline (μg./bone)	
			In medium	In rudiments	In medium	In rudiments
6	10	0·1	12	10	1·9	2·1
	0	0·1	7	18	1·0	2·6
7	10	0·01	16	13	1·9	1·0
	0	0·01	11	20	1·1	1·9
8	5	0·1	18	18	2·8	3·8
	0	0·1	11	28	2·5	4·5

hr./explant, whereas with the paired rudiments grown in the presence of 0·1 μg. of hydrocortisone/ml., the corresponding value was 1·26 μl./hr./explant.

Combined effects of vitamin A and hydrocortisone. In Expt. 6 (in Table 5) 0·1 μg. of hydrocortisone/ml. was found to have some inhibitory influence on the effect of 10 i.u of the vitamin/ml., but the changes produced by the latter agent were well marked though not extreme. The A-hypervitaminotic explants (Plate 1*c*) were much smaller than their paired controls (Plate 1*d*), but were neither fractured nor greatly distorted. Metachromasia largely disappeared from a fairly wide superficial zone and was greatly diminished throughout the middle segment of the shaft. Much metachromatically staining matrix remained in the ends of the explants, however, and had the dense uniform appearance characteristic of that in the (hydrocortisone-treated) controls. The vitamin had its characteristic effect in depressing the amount of hexosamine and hydroxyproline in the rudiment and increasing that in the medium, while the total synthesis was little affected (Table 5).

The rudiments of Expt. 7 were grown in the same medium but with the addition of only 0·01 μg. of hydrocortisone/ml. Vitamin A (10 i.u./ml.) produced much more drastic changes than in the previous experiment. The explants developed fractures and became very flabby. Sections showed extensive loss of metachromasia throughout the cartilage; the remaining metachromatic matrix was less dense and much less heavily stained than in Expt. 6. The matrix of the controls also stained less intensely with toluidine blue than in the previous experiment, and especially in the zones of flattened cells there were signs of some excessive hydration, though less than in controls grown in hormone-free medium. The more drastic histological action in this experiment was not reflected in a greater loss of hexosamine, although there was slightly more effect on the hydroxyproline content than in Expt. 6. The total synthesis of these compounds was not affected by the severe action of the vitamin (Table 5).

The characteristic effects of the vitamin on the components of the matrix were still apparent at 5 i.u./ml. in the presence of 0·1 μg. of hydrocortisone/ml. (Expt. 8 in Table 5).

DISCUSSION

In previous studies, it was found that after 6 days' cultivation on a natural medium containing added vitamin A (10 i.u./ml.) the amino sugar content of cartilaginous limb-bone rudiments was half that of the control rudiments (Dingle *et al.* 1961). This observation has been confirmed in the present investigation in which a synthetic medium has been used. The experiments described in the present paper have shown also that, if the effect of vitamin A on embryonic cartilage is not too severe, the total synthesis of hexosamine and hydroxyproline is not diminished but that, as compared with controls, a greater proportion of both these substances is shed into the medium. This is consistent with the observation by Fell *et al.* (1956) that one of the first effects of excess of vitamin A on cartilage is a decreased incorporation of inorganic sulphate into the intercellular material.

It was surprising to find that the untreated explants cultured on medium BGJ shed considerable quantities of both amino sugars and hydroxyproline into the culture medium. Hydrocortisone, in physiological concentrations, decreased the amount liberated and, for hydroxyproline, correspondingly raised that in the tissue, but the release remained fairly high. Whether this liberation of hexosamines and hydroxyproline is a normal feature of growing cartilage or a peculiarity of organ cultures is not

known. Neither is it known what the hexosamine- and hydroxyproline-containing compounds are, since both of these substances were assayed after acid hydrolysis of the medium. From preliminary work, however, it appears that the hydroxyproline-containing material is of low molecular weight, because over 90% of the hydroxyproline released into the medium by control cultures is diffusible. Jasin, Fink, Wise & Ziff (1962) found that in growing but not in adult animals hydroxyproline is present in the blood. It seems possible that this imino acid may normally be shed from connective tissue during growth, but as yet its source *in vivo* is not known. J. J. Reynolds (personal communication), in experiments with protein-free synthetic media, has confirmed that hydroxyproline is liberated into the medium from 7-day rudiments in untreated cultures, but he found little release of hexosamine. The 6-day rudiments used in our experiments had a dry weight of about 0·07mg./bone, a hydroxyproline content of 0·5–0·6μg./bone and a hexosamine/hydroxyproline ratio 13·0. In contrast, the 7-day rudiments used by Reynolds were nearly 3 times as heavy, contained 1·4μg. of hydroxyproline/bone and had a hexosamine/hydroxyproline ratio 5·0. It is thought that the relatively high proportion of hexosamine in the 6-day rudiments may be associated with a period of active synthesis of this component, and that this may account for the release of a relatively large amount of hexosamine into the culture medium in our experiments.

Even with relatively toxic doses of retinol the total synthesis of hexosamine and hydroxyproline was found to be considerable although it was less than in the control cultures. Thus it appears that the activation of lysosomes, release of acid hydrolases and consequent degradation of connective-tissue matrix that occur under the influence of excess of retinol do not grossly inhibit the synthesis of certain constituents of cartilage matrix. The results of the present investigations are therefore not inconsistent with the observations of Wolf & Varandani (1960), which suggested that vitamin A plays a role in the biosynthesis of polysaccharides. Since synthesis and degradation may occur simultaneously in hypervitaminosis, the net result will depend on the relative rates of the two processes. Thus when high concentrations of retinol (10i.u./ml.) are used, which not only release lysosomal enzymes but also inhibit synthesis of connective-tissue components, a drastic breakdown of cartilage matrix is observed. Differences between the synthetic activities of the epiphyses and the shaft may explain the regional effects of the vitamin. Both in untreated explants in culture (Fell *et al.* 1956) and in the normal embryo (Amprino, 1954) the diaphysial cartilage rapidly loses its capacity to incorporate $^{35}SO_4^{2-}$, which continues to be actively bound by the epiphyses and proliferative zones; it is significant that the shaft is the first region to lose its metachromasia in response to vitamin A.

The balance between the synthesis and degradation of connective tissues may be disturbed not only by factors influencing synthesis but also by variations in the rate of degradation. The latter process might be affected by differences in the activities of lysosomal enzymes and by factors controlling the release of these enzymes from the lysosomal particles. For example, inhibition of lysosomal protease by ϵ-aminohexanoic acid (Ali, 1964) largely prevents the breakdown *in vitro* of the matrix of retinol-treated cartilage (H. B. Fell & J. T. Dingle, unpublished work). Further, hydrocortisone, at relatively high concentrations (7·5μg./ml.), retards the action of excess of vitamin A on embryonic cartilage in culture (Fell & Thomas, 1961); as hydrocortisone stabilizes lysosomal membranes exposed to damaging agents (Weissman & Dingle, 1961; Weiss & Dingle, 1964), it seems possible that the steroid diminishes the rate of degradation of cartilage matrix by inhibiting the release of lysosomal enzymes.

In contrast with the action of hydrocortisone in high concentrations the physiological quantities of the hormone used in the present experiments did not materially affect the action of excess of retinol on the limb-bone rudiments. Schryver (1965) has shown that low doses of hydrocortisone prevent the abnormal hydration of cartilage grown in chemically defined media that was observed by Biggers (1963). We have confirmed both these findings, but no satisfactory explanation of this action of hydrocortisone is yet available. Our observations suggest that hydrocortisone, at a physiological concentration, may be a desirable component of chemically defined media in biochemical studies on the action of other physiologically active compounds.

H. B. F. is a Royal Society Research Professor. J. T. D. and J. A. L. are members of the Medical Research Council's external staff.

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The Precursors of the Xylene Ring in Riboflavine

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(*Received* 21 *June* 1965)

1. The nature of the precursors of the xylene ring in riboflavine was reinvestigated with growing as well as resting cells of *Eremothecium ashbyii*. 2. The incorporation of acetoin into riboflavine was very low; further, [2-^{14}C]pyruvate and [1-^{14}C]acetate were equally effective as precursors of lumichrome, and pyruvate was much more active as a precursor of acetoin. These results exclude acetoin as a direct precursor of riboflavine. 3. Addition of unlabelled glucose decreased the incorporation of [^{14}C]acetate into riboflavine more than it decreased the conversion of acetate into carbon dioxide, indicating that acetate is not a direct riboflavine precursor. 4. The incorporation of various sugars and dilution experiments suggest that a derivative of the intermediates of the pentose phosphate cycle is the precursor of the xylene ring in riboflavine.

Masuda (1956) isolated a pteridine derivative, 6,7-dimethyl-8-ribityl-lumazine, from cultures of *Eremothecium ashbyii*, an organism active in the synthesis of riboflavine. On structural grounds and on the basis of chemical studies this author suggested that acetoin (3-hydroxy-2-butanone) was the precursor of the aromatic ring in riboflavine (ring A). This hypothesis was further strengthened by the finding of Goodwin & Treble (1958) that [1-^{14}C]acetoin was incorporated into riboflavine better than [2-^{14}C]acetate in whole cells of *E. ashbyii*. The more recent studies of Plaut (1963) show that a molecule of 6,7-dimethyl-8-ribotyl-lumazine donates a 'four carbon unit' to another molecule leading to the formation of riboflavine and a derivative of 4,5-diaminouracil. This finding, however, does not exclude the possibility that acetoin is the ultimate precursor of the four carbon atoms in 6,7-dimethyl-8-ribotyl-lumazine and hence of the aromatic ring of riboflavine. Kishi, Asai, Masuda & Kuwada (1959) presented evidence that cell-free preparations of *E. ashbyii* could bring about the condensation of acetoin with 4-aminoribotyl-5-aminouracil to form 6,7-dimethyl-8-ribotyl-lumazine.

The earlier studies of Plaut (1954), however, show that both the carboxyl and methyl carbon atoms of acetate are incorporated into the aromatic ring of riboflavine. This is a strong evidence against acetoin being involved in this biosynthesis. Barring the direct reduction of acetate to acetaldehyde, a reaction known to occur in some anaerobic organisms only, the only known mechanisms of acetoin biosynthesis involve the loss of the carboxyl group of acetate. Klungsøyr (1954), working with non-growing cells of *E. ashbyii*, showed very poor incorporation of carboxyl-labelled acetate into riboflavine. We have attempted to compare the incorporation of acetate, acetoin and pyruvate (a direct precursor of acetoin and acetate) into riboflavine in both growing and standing cultures of *E. ashbyii*. We also measured the effect of adding non-radioactive diluents.

Our results in general show poor incorporation of pyruvate, acetate and acetoin into riboflavine. This, taken together with the dilution experiments, suggests that none of these compounds is directly involved in the synthesis of riboflavine ring A. By using various labelled sugars we obtained results which lend support to the hypothesis that a derivative of the pentose phosphate-cycle intermediates may act as the ultimate precursor of the xylene ring in riboflavine.

MATERIALS AND METHODS

Biological material. Eremothecium ashbyii (American Type Culture Collection no. 6747) was maintained on agar plates of the medium used for growth and transferred periodically.

Chemicals. Radioactive chemicals were purchased from The Radiochemical Centre (Amersham, Bucks.). Other chemicals, unless otherwise indicated, were chemically pure or B.P. products from various commercial companies.

Growth conditions. Initially cells were grown with large inocula (5%) in an aqueous medium that contained (per litre): yeast extract (Difco), 2g.; Bacto peptone (Difco), 5g.; a centrifuged 25% (w/w) solution of corn-steep liquor (A. E. Staley Manufacturing Co., Decator, Ill., U.S.A.), 20ml.; glucose, 40g.

The final pH of the medium was 5·6. Cells were grown

with continuous shaking in flasks filled to 10–20% of their capacity. The temperature was maintained at 29±1°.

Collection of mycelia. Twenty-four-hours-old cultures were filtered through muslin under suction and washed with cold saline. The resulting mycelial pad could then be peeled off the cloth and weighed directly. The cells were suspended in Krebs–Ringer phosphate buffer containing 10mg. of guanine/100ml. for experiments with non-growing cells. The manner of incubation with radioactive compounds is described in the Results section.

Extraction of riboflavine. The incubation was stopped by acidifying to pH1 with conc. HCl. The suspension was then autoclaved for 20min. at 15lb./in^2. To the resulting hydrolysate enough Na_2SO_4 was added to saturate the solution. Riboflavine was extracted three times with 1vol. of benzyl alcohol. At this stage benzyl alcohol forms an emulsion with the aqueous hydrolysate. The emulsion was easily broken by centrifugation and the upper benzyl alcohol layer pipetted out. The benzyl alcohol extracts were pooled, diluted with 4vol. of ether and the riboflavine was twice extracted with 5ml. of water.

Column chromatography of riboflavine. Florisil (60–100 mesh) (Floridin Co., Warren, Pa., U.S.A.) was suspended in 2% (v/v) acetic acid. When the earth settled, the slightly turbid supernatant fluid was decanted. The earth was resuspended in the 2% acetic acid and packed as a slurry in columns 1·5cm. diam. and 10cm. high. The aqueous extract of riboflavine was placed directly on the Florisil column. The column was washed with 500ml. of 2% (v/v) acetic acid and the yellow band was eluted with 20% (v/v) pyridine.

Estimation of riboflavine. Riboflavine was measured by the intensity of its yellow colour at 445mμ by using the millimolar extinction coefficient of 11·3. The extinction coefficient of riboflavine was checked with an authentic sample of riboflavine (Eastman Kodak Co.).

Crystallization of riboflavine to constant specific activity. After assaying the initial amount of riboflavine eluted from the columns, 25mg. of non-labelled riboflavine was added and the solution evaporated to dryness. The riboflavine was crystallized to constant counts/min./μmole of the riboflavine, from the following solvents: 10% (w/v) acetic acid, 70% (v/v) propanol and conc. HCl diluted with water (4–5 vol.). One crystallization from each solvent was always enough to achieve constant specific activity.

Preparation of lumichrome. Lumichrome was prepared by the procedure of Karrer, Salomon, Schöpp, Schlitter & Fritzsche (1934). A dilute solution of purified riboflavine containing 20mg./100ml. was prepared in 50% (v/v) methanol. The solution was placed in Erlenmeyer flasks of 250ml. capacity and irradiated (three fluorescent lamps, 15w each) for 72hr. During this period the intense yellow colour of the solution turned pale green. The methanolic solution was evaporated to dryness *in vacuo* and the yellow material was taken up in two 3ml. portions of 0·1N-NaOH. The alkaline solution was shaken once with 50ml. of chloroform and the chloroform discarded. The alkaline liquid was brought to pH2 with conc. HCl and the lumichrome extracted five times with 50ml. of chloroform.

Chromatographic purification of lumichrome. A portion (10g.) of Celite 545 (L. Light and Co. Ltd., Colnbrook, Bucks.) was triturated in a mortar with 4ml. of water and packed tightly into a column (1·5cm. diam.). The combined chloroform extract was passed through the column and washed with an additional 50ml. of chloroform saturated with 0·1N-HCl. The lumichrome passed through the column, leaving a band of yellow impurities on top of the column.

To test the purity of lumichrome, it was chromatographed on Whatman no. 1 filter paper in the different solvent developed by Whitby (1952). In all cases it gave a single spot which corresponded in R_F to the five-times-crystallized lumichrome prepared on a large scale by the procedure of Karrer *et al.* (1934).

However, the recrystallized material prepared by the method of Karrer *et al.* (1934) was not pure and on chromatography on paper it gave two spots. Spectral analysis of these spots showed that lumichrome was contamined with traces of riboflavine. Therefore the whole material was purified by passing it through Celite columns and recrystallizing once more from acetic acid.

Estimation of lumichrome. The chromatographically pure material was used as a standard. Complete spectra were taken in 0·5N-NaOH and in 0·1N-HCl. The shape of these spectral curves agreed with those reported by McNutt (1954). The molar extinction coefficients were calculated at different wavelengths on this sample on the basis of the nitrogen content of the material, as determined by a micro-Kjelbahl method. These values are reported in Table 1. For routine analysis lumichrome was estimated from the extinction at 340 and 425mμ of its solutions in 0·5N-NaOH.

Isolation of acetoin from mycelial cultures. Whenever incorporation of radioactivity into acetoin was to be determined, the following procedure was followed. To a suitable portion of the cell suspension 50mg. of unlabelled acetoin was added and the final volume made up to 50ml. with water. The mixture was distilled *in vacuo* almost to dryness. The clear distillate contained the acetoin.

Conversion of acetoin into 2,4-dinitrophenylosazone. 2,4-Dinitrophenylhydrazine (1·5g. dissolved in 50ml. of 30%, w/v, perchloric acid) was mixed with the distillate containing the acetoin and the mixture refluxed for 6hr. on a water bath. The voluminous precipitate of the osazone was filtered off on a Buchner funnel, washed with 10ml. of water and dried at 50°.

Crystallization of osazone to constant specific activity. The osazone was crystallized alternately from aniline and nitrobenzene until the radioactivity per milligram of osazone became constant. In one case, after the osazone had been crystallized several times from the above solvents, it was recrystallized from several litres of acetic acid. The recrystallized material showed no change in specific radioactivity.

Organic synthesis of 3-hydroxy[1-^{14}C]butan-2-one (acetoin). The procedure of Brady, Rabinowitz, van Baalen & Gurin (1951) was used to synthesize acetoin except that the synthesis was scaled down to 10m-moles of lactonitrile.

Table 1. *Molar extinction coefficient of lumichrome*

	In 0·1N-HCl		In 0·5N-NaOH		
Maxima (mμ)	262	350	265	340	425
10^{-3} $E_{max.}$ (cm.^{2} mole^{-1})	33·3	8·81	40·3	6·50	6·95

Radioactive purity of acetoin. For the determination of the radioactive purity of acetoin as well as for further purification, the acetoin was chromatographed on a Celite column. A similar column was used by Neish (1950) to separate acetoin, diacetyl and butane-2,3-diol.

A portion (45g.) of Celite 545 was wetted with 20ml. of water and triturated thoroughly in a mortar. It was packed in a column of 2cm. diam. The radioactive acetoin (0·4g. of total synthesized product) was taken up in 2ml. of water triturated with 5g. of Celite and packed on the top of the column. Acetoin was eluted with ethyl acetate saturated with water and 10ml. fractions were collected. Acetoin was determined colorimetrically and the radioactivity was estimated on the same sample. The profile obtained shows that acetoin comes out in the first few fractions of eluate and the curve of radioactivity corresponds to the curve of acetoin concentration.

The acetoin was recovered from ethyl acetate by diluting it with 2vol. of normal pentane, and re-extracting with water. The aqueous extract was shaken again with normal pentane to extract the residual ethyl acetate. The aqueous acetoin was preserved at −12°.

Colorimetric estimation of acetoin. For rapid determination of acetoin a modification of the procedure of Happold & Spencer (1952) was used. A sample containing 20–100μg. of acetoin was mixed with 5ml. of 0·1% (w/v) 2,4-dinitrophenylhydrazine dissolved in 5% (w/v) perchloric acid. It was placed in a tube tightly closed with a rubber stopper and boiled for 1hr. in a water bath. After cooling, a pinch of kaolin was added to the tube, which was centrifuged. The supernatant was discarded and the precipitate was washed once with dilute HCl and once with water. The precipitate was extracted twice with 5ml. of sodium ethylate. The extinction of the violet colour was read in a Klett photometer with a 540mμ filter. The value was compared against a standard containing 40μg. of acetoin. The colour intensity is linear within the range 20–100μg. of acetoin. (Sodium ethylate is prepared by dissolving 3g. of clean sodium in 1000ml. of absolute ethanol.)

Determination of radioactivity. All samples were plated on preweighed glass planchets and counted on a gas-flow counter (Nuclear–Chicago Corp.) in which 1μc gives 600000 counts/min. The planchets were reweighed and whenever applicable corrections for self-absorption were applied.

The samples were counted for 10min. If activities of less than 10 counts/min. were obtained the counting was continued for an additional 10min. The background count was about 18 counts/min.

RESULTS

Incorporation of [1-^{14}C]*acetate and* [2-^{14}C]*pyruvate into acetoin in growing cells.* Pyruvate and acetaldehyde are the only known metabolic precursors of acetoin. The label from carboxyl-labelled acetate metabolized through the tricarboxylic acid cycle will appear in pyruvic acid in the carboxyl group only, and therefore will not label acetoin. The direct reduction of acetate to acetaldehyde is known to occur in some anaerobic organisms only. The only other known route through which carboxy-labelled acetate can label acetoin would involve its incorporation into glucose, via carboxyl-labelled pyruvic acid, the randomization of the isotope in the glucose through the hexose monophosphate shunt, the subsequent production of pyruvate and its conversion into acetoin. Such a route would involve great dilutions. As carboxyl-labelled acetate is known to be incorporated into riboflavine, it was necessary to find the relative magnitude of the incorporation of acetate and pyruvate into acetoin in *E. ashbyii*.

Two flasks each containing 400ml. of a 24hr.-old culture were incubated with 1μmole containing 10^6 counts/min. of either [1-^{14}C]acetate or [2-^{14}C]pyruvate (the direct precursor of [1-^{14}C]acetate). After 12hr. the incubation was stopped by adding 5ml. of conc. hydrochloric acid. Acetoin was isolated as the 2,4-dinitrophenylosazone and recrystallized four times alternately from aniline and nitrobenzene. Constant specific activity was obtained after the first crystallization. The results showed specific activities 40 and 255 counts/min./μmole for the acetoin produced from [1-^{14}C]acetate and [2-^{14}C]-pyruvate respectively.

Incorporation of acetate and pyruvate into riboflavine and acetoin in standing cultures. To obtain a correlation between the labelling of riboflavine and acetoin by acetate and pyruvate the incorporation of these substrates into both acetoin and riboflavine was studied simultaneously in standing cultures. Cell suspensions of *E. ashbyii* in Krebs–Ringer phosphate buffer containing guanine were incubated with [2-^{14}C]pyruvate, [1-^{14}C]acetate and [2-^{14}C]-acetate with or without non-radioactive diluents. At the end of 12hr. the reaction was stopped and the incubation medium was divided into two equal portions: one portion was used to isolate riboflavine; to the other portion 50mg. of unlabelled acetoin was added and the osazone prepared and crystallized to constant specific activity (Table 2). Though [2-^{14}C]pyruvate was incorporated about 60 times as effectively as [1-^{14}C]acetate into acetoin its incorporation into riboflavine was less than one and a half times as effective as the incorporation of [1-^{14}C]acetate. Comparing [2-^{14}C]pyruvate with [2-^{14}C]acetate, the incorporation of [2-^{14}C]acetate was about three times that of the pyruvate into riboflavine whereas it labelled acetoin less than one-third as effectively as pyruvate.

The addition of 15 molar equiv. of unlabelled acetate to incubations with labelled pyruvate and vice versa showed very little effect.

Incorporation of acetate, pyruvate and acetoin into riboflavine in standing cultures. In this experiment 5% cell suspensions of *E. ashbyii* in Krebs–Ringer phosphate buffer containing guanine were incubated with [1-^{14}C]acetate, [2-^{14}C]acetate, [2-^{14}C]-pyruvate and 3-hydroxy[1-^{14}C]butan-2-one (radioactive acetoin). Because of the larger endogenous dilution expected with pyruvate and acetate, the

Table 2. *Incorporation of acetate and pyruvate into riboflavine and acetoin*

Each flask contained 17 μmoles of the radioactive substrate (10×10^6 counts/min.), 50ml. of a 5% (w/v) suspension of washed *E. ashbyii* cells in Krebs–Ringer phosphate buffer, 5mg. of guanine and, whenever indicated, 250 μmoles of unlabelled substrate. The flasks were incubated for 12hr. at 30° on a shaker incubator (100 strokes/min.). The reaction was stopped by adding 1·5ml. of conc. HCl.

		Riboflavine		Acetoin	
Labelled substrate	Unlabelled substrate	μmoles formed	Counts/min./μmole	μmoles formed	Counts/min./μmole
[1-^{14}C]Acetate	—	2·0	375	560	38
[1-^{14}C]Acetate	Pyruvate	2·1	280	720	31
[2-^{14}C]Acetate	—	2·6	1450	570	600
[2-^{14}C]Acetate	Pyruvate	2·5	1500	575	630
[2-^{14}C]Pyruvate	—	2·8	560	560	2130
[2-^{14}C]Pyruvate	Acetate	2·3	413	570	2700

Table 3. *Incorporation of acetoin, acetate and pyruvate into lumichrome and carbon dioxide*

Results presented are the averages of quadruplicate flasks. Each flask was provided with a suspended well containing 1ml. of N-NaOH and contained 15ml. of 5% (w/v) cell suspension in Krebs–Ringer phosphate buffer with 10mg. of guanine/100ml. Flasks were incubated at 30° for 12hr. The reaction was stopped by the addition of 0·3ml. of conc. HCl. The incubation was continued for an additional 1hr. to trap the liberated CO_2. The riboflavine was isolated from the incubation and degraded as described in the Materials and Methods section.

	Riboflavine		
Labelled substrate	μmole formed	Counts/min./μmole of lumichrome	Percentage converted into CO_2
[1-^{14}C]Acetoin	0·50	19	0·24
[2-^{14}C]Pyruvate	0·53	192	4·4
[2-^{14}C]Acetate	0·24	510	6·11
[1-^{14}C]Acetate	0·83	125	28·0

specific activity of the added acetate and pyruvate ($6{\cdot}67 \times 10^4$ counts/min./μmole) was made higher than the specific activity of the added acetoin ($1{\cdot}25 \times 10^4$ counts/min./μmole). Additional flasks were also incubated with radioactive substrate combined with non-labelled diluent.

The liberated carbon dioxide was trapped in 1N-sodium hydroxide placed in a well suspended from the stopper of the flask. Riboflavine was isolated and crystallized to constant specific activity. The purified riboflavine was converted into lumichrome. Lumichrome was purified and assayed for its radioactivity. The average results of this experiment are in Table 3. The replicates in this experiment agreed within 30% of the mean.

These results indicate that acetoin was poorly metabolized and poorly incorporated into riboflavine. The 19 counts/min./μmole are of marginal significance and can possibly be due to counting errors as the amount counted in all cases was about 10% of the total riboflavine formed.

The addition of unlabelled diluents (250 μmoles) of acetate or pyruvate to the flasks containing radioactive acetoin did not significantly change the incorporation. Similarly the addition of unlabelled acetoin or acetate to the flasks containing the labelled pyruvate and the addition of unlabelled pyruvate and acetoin to the flasks containing the radioactive acetate did not alter the incorporation drastically.

Effect of glucose on the incorporation of acetate into riboflavine and carbon dioxide. During the course of experiments with growing cells it was observed that when sufficient glucose was present in the culture medium the incorporation of acetate or pyruvate into riboflavine was exceedingly poor. Therefore the effect of glucose was examined in two experiments.

In Expt. 1 cells were grown as described previously and samples of the culture were withdrawn aseptically at intervals for glucose analysis. After 48hr. incubation the concentration of glucose fell below 1·0mg./100ml. At that point 100ml. portions of the culture were transferred to two sterile flasks. One flask was incubated with 20 μmoles of [2-^{14}C]-acetate ($1{\cdot}5 \times 10^6$ counts/min.) alone, and the other with the same [2-^{14}C]acetate with the addition of 2g. of unlabelled glucose. During incubation the flasks were aerated with carbon dioxide-free air and the evolved carbon dioxide was trapped in 1N-sodium hydroxide. The incubation was stopped after 12hr. Riboflavine was isolated, crystallized to constant specific activity and counted. It was further degraded to lumichrome, which was purified and counted. The results are shown in Table 4, Expt. 1.

In Expt. 2 cells were grown for 24hr., harvested

and resuspended with 19 vol. of Krebs–Ringer phosphate buffer containing guanine. Portions (100 ml.) of the suspension were incubated with 10 μmoles of the labelled substrate containing 2×10^6 counts/min. In certain flasks (as indicated) 2500 μmoles of glucose or 5000 μmoles of sodium pyruvate were added; the incubation time was 6 hr., after which the radioactivity of carbon dioxide, riboflavine and lumichrome were determined as described in the Materials and Methods section. The results of this experiment are shown in Table 4, Expt. 2.

It is clear from these experiments that the presence of glucose inhibited the incorporation of acetate into the lumichrome to values below 10% of the control value. This inhibition seems to be of the same magnitude in growing and resting cells. The conversion of acetate into carbon dioxide was also inhibited but to a much smaller degree. The addition of amounts of pyruvate equivalent to the glucose had much less effect on the incorporation of acetate into lumichrome. It is also noted that [1-^{14}C]glucose and [6-^{14}C]glucose are better incorporated into lumichrome than acetate.

A calculation of the ratio of the isotope in lumichrome to the isotope in riboflavine shows that the lumichrome part of the riboflavine molecule contained 50–56% of the isotope when [2-^{14}C]acetate was used, under vastly divergent conditions.

Incorporation of monosaccharides into riboflavine. In the previous experiment the addition of glucose was shown to dilute the incorporation of acetate

Table 4. *Incorporation of acetate and glucose into riboflavine and carbon dioxide*

See text for experimental details

	Labelled substrate	Unlabelled substrate	Riboflavine: μmoles formed	Riboflavine: Counts/min./μmole of riboflavine	Riboflavine: Counts/min./μmole of lumichrome	Percentage converted into CO_2
Expt. 1 (growing cells)	[2-^{14}C]Acetate	—	13·5	900	483	34
	[2-^{14}C]Acetate	Glucose	16·1	59	30	20
Expt. 2 (non-growing cells)	[2-^{14}C]Acetate*	—	2·74	735	368	59
	[2-^{14}C]Acetate	Glucose	2·12	59	33	16
	[2-^{14}C]Acetate*	Pyruvate	1·19	522	289	60
	[6-^{14}C]Glucose*	—	3·02	1412	522	24
	[U-^{14}C]Glucose*	—	2·60	1307	503	44

* Average of duplicate experiments, which agreed in each case to better than 30% of the mean.

Table 5. *Incorporation of various sugars into riboflavine and carbon dioxide*

See text for experimental details.

	Labelled substrate	Unlabelled substrate	Riboflavine: μmoles formed	Riboflavine: Counts/min./μmole of riboflavine	Riboflavine: Counts/min./μmole of lumichrome	Percentage converted into CO_2
First batch	[1-^{14}C]Glucose*	—	1·27	1995	1194	33·5
	[6-^{14}C]Glucose*	—	1·41	2320	843	26·4
	[6-^{14}C]Glucose*	Ribose	1·54	1920	893	26·0
	[1-^{14}C]Ribose	—	1·65	3360	2300	7·2
	[1-^{14}C]Ribose	Glucose	1·38	725	486	
	[U-^{14}C]Erythritol	—	1·58	126	148	0·16
	[U-^{14}C]Erythritol*	Glucose	1·18	62	118	0·04
Second batch	[U-^{14}C]Glucose*	—	1·08	2526	1071	19·4
	[U-^{14}C]Glucose*	Xylose	1·93	1900	584	20·3
	[U-^{14}C]Glucose	Glycerol	1·24	2730	800	25·3
	[U-^{14}C]Ribose*	—	1·34	2154	989	3·6
	[U-^{14}C]Xylose	—	1·04	510	302	0·68
	[U-^{14}C]Xylose	Glucose	2·14	239	275	0·32
	[U-^{14}C]Glycerol*	—	1·52	2434	1120	27·5

* Results are averages of duplicate flasks, which agree in all cases to better than 25% of the mean.

into the riboflavine more than the dilution by pyruvate. Glucose was also shown to be a more efficient precursor of the xylene ring in riboflavine.

The following experiment was designed to test the effectiveness of various monosaccharides and derivatives as precursors of the xylene ring of riboflavine. Because of the large number of flasks involved, the experiment was run in two separate batches.

E. ashbyii cells were grown for 24hr. with aeration, harvested and resuspended in 19vol. of Krebs–Ringer phosphate buffer containing 10mg. of guanine/100ml. Each flask contained 100ml. of the cell suspension, 10μmoles of radioactive substrate (2×10^6 counts/min.) and, whenever indicated, 2500μmoles of non-radioactive diluent substrate (5000μmoles for glycerol diluent). Incubation time was 6hr. The isolation of carbon dioxide, riboflavine and lumichrome was as described in the Materials and Methods section.

The results of this experiment are presented in Table 5. Ribose was incorporated as efficiently as glucose, although its utilization (measured by conversion into carbon dioxide) is much poorer. The incorporation of xylose is poor but its conversion into carbon dioxide is very low whereas the incorporation of glycerol into carbon dioxide and riboflavine is similar to that of glucose.

The production of radioactive carbon dioxide from glucose 1-phosphate and glucose 6-phosphate is similar as is their incorporation into lumichrome.

DISCUSSION

Both acetate and acetoin have been suggested as the ultimate precursors of the xylene ring (ring A) in riboflavine. Plaut (1961), on the basis of the incorporation of [1-^{14}C]acetate into riboflavine, favours the acetate hypothesis. However, Goodwin (1963), basing his arguments on structural grounds, on the incorporation of acetoin into riboflavine (Goodwin & Treble, 1958) and on the higher degree of randomization occurring in the incorporation of [2-^{14}C]acetate compared with the incorporation of [1-^{14}C]- or [6-^{14}C]-glucose into the xylene ring of riboflavine (Plaut, 1954), advances the acetoin hypothesis.

We attempted to resolve this problem by studying the incorporation of radioactive substances in recrystallized riboflavine and in chromatographically pure lumichrome obtained therefrom. Our purification procedures led to a low recovery of lumichrome, which makes further degradation studies impractical. We assumed in all cases that the activity in lumichrome was essentially all in the xylene ring. This is justified since Plaut's (1954) results show very low incorporation of acetate and glucose in the pyrimidine ring of riboflavine (ring C). Further, we ran all our incubations in the presence of large amounts of purines, which Goodwin & Jones (1956) have shown to decrease the incorporation of serine, a more direct precursor of the pyrimidine ring, to very low values.

Our results provide evidence against the involvement of either acetoin or acetate as the ultimate precursor of the xylene ring in riboflavine. The evidence against acetoin can be summarized as follows.

1. The very low incorporation of acetoin into riboflavine (Table 3). These results are contrary to those reported by Goodwin & Treble (1958). The reason for the difference is not clear as these authors have not yet reported the details of their experiments.

2. The incorporation of [2-^{14}C]pyruvate into lumichrome was not better than the incorporation of its direct product [1-^{14}C]acetate, whereas [2-^{14}C]-pyruvate was more effective than [1-^{14}C]acetate as a precursor of acetoin, the ratio being 60:1 in standing cultures and 6:1 in growing cells (see Table 2).

3. [2-^{14}C]Acetate was incorporated about one-fifth as effectively as [2-^{14}C]pyruvate into acetoin, whereas its incorporation into lumichrome was 3 to 5 times as effective (Table 2).

4. The lower dilution of the incorporation of acetate into lumichrome by unlabelled pyruvate (a direct precursor of acetoin) compared with the dilution by unlabelled glucose (Table 4). It is not clear why pyruvate did not dilute the degradation of acetate into carbon dioxide; possibly it stimulates the tricarboxylic acid cycle to offset the dilution.

These last three points we take to show that the poor incorporation of acetoin is not due solely to a difficulty in its entry into the cells.

The evidence against acetate can be summarized as follows:

1. The higher degree of randomization of the isotope from acetate than the isotope from glucose in the xylene ring of riboflavine as shown by Plaut (1954) and pointed out by Goodwin (1963).

2. The very large dilution of the incorporation of acetate into lumichrome by non-radioactive glucose. This dilution is greater than the dilution of the degradation of acetate into carbon dioxide by glucose. It is also larger than the dilution of acetate incorporation into lumichrome by unlabelled pyruvate. These two facts show that the dilution of the incorporation of acetate into lumichrome is more than can be accounted for by the dilution of the specific activity of the radioactive acetate by acetate units formed from unlabelled glucose.

We would like to suggest as a tentative alternative hypothesis that a derivative of the intermediates of the pentose phosphate cycle acts as the

precursor of the xylene ring in riboflavine. The evidence favouring such a hypothesis can be summarized as follows.

1. The high amount of incorporation of ribose into riboflavine, especially if this is taken in conjunction with the poorer utilization of ribose than of glucose as measured by its conversion into carbon dioxide. The incorporation of xylose, though poorer than glucose, may be explained by its low degree of activation or entry into the cell. However, if the ratio of incorporation into lumichrome to oxidation to carbon dioxide is calculated (Table 5), ribose and xylose show almost equal values, which are approximately tenfold those obtained for glucose or glycerol.

2. The similar incorporation of isotope into the ribitol side chain and the lumichrome portion of the riboflavine molecules from various sources and under various conditions (Tables 3, 4 and 5), suggesting a common precursor of the xylene ring and the ribitol side chain which is presumably synthesized through a pentose (Plaut, 1961).

A similar isotope distribution has been observed by Plaut (1954) and Plaut & Broberg (1956). The evidence cited against acetate above and evidence 4 cited against acetoin can also be taken to favour this hypothesis.

The labelling pattern in riboflavine obtained from labelled acetate (Plaut, 1954) also fits this hypothesis if a four carbon atom sugar is incorporated into riboflavine, and if the major part of the pentose-cycle intermediates are produced through the transketolase, transaldolase reactions.

The observation that unlabelled glucose effectively dilutes the incorporation of pentoses into riboflavine while the reverse dilution is less pronounced may be a reflexion of the limited ability of the cells to absorb or activate pentoses. But it is not clear why xylose had a more marked effect than ribose on the incorporation of glucose into lumichrome.

As cells are not permeable to phosphate esters the direct testing of pentose-cycle intermediates as precursors of the xylene ring of riboflavine will have to await the preparation of cell-free extracts capable of affecting the synthesis of 6,7-dimethyl-8-ribotyl-lumazine.

The authors are grateful to Dr I. F. Durr for many useful discussions in the course of the work and during the preparation of the manuscript, and to Mr Souren Chekijian for very able technical assistance. This report is in part taken from a Thesis submitted by S. N. A. to the American University of Beirut in partial fulfilment of the requirement for the degree of M.Sc. The work was supported in part by grant A1-03773 from the National Institute of Allergy and Infectious Diseases of the National Institutes of Health, U.S.A.

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Absorption Spectra of Sperm-Whale Ferrimyoglobin

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(*Received* 3 *June* 1965)

1. Sperm-whale ferrimyoglobin was found to contain 0·308% of Fe, on a dry weight basis, corresponding to a molecular weight of 18130. The solid takes up moisture to an equilibrium state, and was then assayed to contain 0·280% of Fe. 2. Absorption spectra are presented for acidic ferrimyoglobin, $Fe^{+}(H_2O)$, and its conjugate base, Fe–OH, as well as for the fluoride and cyanide complexes, within the range 200–2500mμ. Data for ferromyoglobin–carbon monoxide, Fe(II)–CO, in the visible region are also included. 3. Minor spectral differences have been found between whale and horse myoglobins, particularly in the effect of temperature on the visible-absorption spectrum of Fe–OH.

Comparison of the physicochemical behaviour of a protein in solution with predictions based on its known crystal structure can best be made for sperm-whale ferrimyoglobin. The X-ray diffraction studies of Kendrew *et al.* (1961) provide the necessary structural data. However, the corresponding physicochemical work on the protein in solution has been carried out mainly on ferrimyoglobin from horse-heart muscle. A systematic kinetic and thermodynamic study of the reactions of sperm-whale ferrimyoglobin in solution would be of value in two ways. It would show to what extent the reactivity of the protein accords with its known crystal structure, and would also make possible the assessment of second-order differences in the properties of myoglobins from different species with their differing basic protein structures. For such studies on solutions, detailed and reliable spectral data are needed, since much of the work involves spectrophotometric techniques. We have therefore obtained, by a standardized and independently calibrated procedure, full absorption spectra for sperm-whale ferrimyoglobin and its common derivatives.

MATERIALS AND METHODS

Sperm-whale ferrimyoglobin was purchased as a freeze-dried salt-free solid from Seravac Laboratories (Maidenhead, Berks.). This material has been shown by E. S. Awad & A. B. Edmundson (personal communication) to be chromatographically identical, on Amberlite IRC-50, with the material used by Edmundson & Hirs (1962) in their studies of amino acid sequence, and by Kendrew *et al.* (1961) in their crystallographic work. The present work was carried out on the Seravac material without further purification.

The iron content of sperm-whale ferrimyoglobin was determined with and without prior drying. The procedure for dry weight analysis involved drying about 5mg. of myoglobin in an oven at 105° for several hours, followed by taking the weight, W_t, of the sample on an electrobalance (Cahn Instrument Co., Paramount, Calif., U.S.A.; precision to ±0·1μg.) at successive intervals of time from about $t = 20$sec. to constant 'equilibrium' weight, W_e, which is attained after 20–30min. The dry weight was obtained by linear extrapolation of $\log(W_e - W_t)$ to zero time. Samples of the undried solid were weighed in air in the normal manner. In both cases iron determinations were made by an improved phenanthroline micro method (Cameron, 1965). By using iron-free reagents, the method was standardized by calibration against Mohr's salt, $Fe(NH_4)_2(SO_4)_2,6H_2O$, and Oesper's salt, $Fe(CH_2NH_3)_2(SO_4)_2,4H_2O$. The method was precise to about 1 in 300.

Absorption spectra were measured at 25° (and in some cases also at 4° and 40°), within the wavelength range 200–2500mμ with about 300 experimental points. All readings were taken in 10mm. cells against a reagent blank. The spectra at 25° were taken on a Zeiss PMQII spectrophotometer. At other temperatures, the spectra were taken on a Unicam SP.500 spectrophotometer in which the temperature of the cell compartment was controlled by circulating water from a thermostatically controlled bath. Concentrations of myoglobin in solution were determined with reference to the ferrimyoglobin–cyanide complex, for which the following molar extinction coefficients ($M^{-1}cm.^{-1}$) were obtained by independent duplicate iron determinations: $10{\cdot}7\times10^3$ at 540mμ; 110×10^3 at 423mμ.

Acidic ferrimyoglobin, $Fe^{+}(H_2O)$, was examined at pH 6·0 and I 0·02; its conjugate base, Fe–OH, at pH 11·5 and I 0·05; the cyanide complex, Fe–CN, at pH 7·0 and I 0·05; the fluoride complex, Fe–F, at pH 6·0 in 1·5M-KF (measurements of the equilibrium constant showed that formation of the complex was 98% complete under these conditions; readings were accordingly corrected from 98 to 100% conversion into Fe–F). The ferromyoglobin–carbon monoxide complex, Fe(II)–CO, was prepared by

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passing pure CO through a solution containing ferrimyoglobin in buffer at pH7·0 with a trace of $Na_2S_2O_4$ added, and extinctions were then measured rapidly. Buffer solutions of pH6 and 7 were 10mM-NaH_2PO_4–NaOH mixtures, and pH11·5 was 10mM-Na_2HPO_4–NaOH mixture, ionic strengths being adjusted with NaCl. All reagents were of A.R. grade, and deionized glass-redistilled water was used throughout.

RESULTS AND DISCUSSION

The results of four determinations of iron in sperm-whale ferrimyoglobin are given in Table 1. The iron content is 0·308 (±0·001)% on a dry weight basis. This corresponds to a molecular weight of 18130 and is within 2% of the value 17816 reported by Edmundson & Hirs (1962) from their amino acid analysis of the protein.

The data in Table 1 also show that when dried ferrimyoglobin is exposed to air it takes up moisture to a reproducible equilibrium state in which about 10% of water is incorporated. The iron content calculated by extrapolation to this equilibrium condition is constant at 0·280 (±0·001)%. This latter result is also obtained, within the same limits of uncertainty, from direct iron determination on the undried stock myoglobin.

The absorption spectra of four myoglobin derivatives are shown in Figs. 1 and 2, and the main band characteristics are summarized in Table 2, which also includes data for Fe(II)–CO in the visible region of the spectrum. Several points arise on comparing these spectra of whale myoglobin with those of horse myoglobin (Hanania, 1953). As expected, their general characteristics are similar. In fact, the cyanide complexes of the two species have nearly identical spectra, which are insensitive to changes in pH, ionic strength or temperature (from 4° to 40°). This presumably implies that strong low-spin bonding at the haematin iron is not favourable to the transmission of charge and 'allied effects' between protein and haem.

On the other hand, minor species differences are observed with the two high-spin compounds $Fe^+(H_2O)$ and Fe–F. Thus whale $Fe^+(H_2O)$ shows 3% stronger absorption in the visible region, and 4–5% weaker absorption in the near ultraviolet, relative to horse $Fe^+(H_2O)$, and whale Fe–F is 4–9% weaker in the visible and 2% stronger at the Soret peak, the protein bands being equal. More-

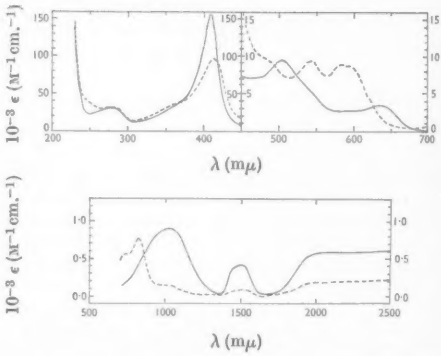

Fig. 1. Absorption spectra of sperm-whale ferrimyoglobin: ——, acidic ferrimyoglobin, $Fe^+(H_2O)$, at 25° and at pH6·0 and I 0·02; – – – –, alkaline ferrimyoglobin, Fe–OH, at 25° and at pH11·5 and I 0·05.

Table 1. *Determination of iron in sperm-whale ferrimyoglobin*

Column (A), weight (mg.) of dried sample, by extrapolation; (B), weight (mg.) of dried sample after equilibration in air; (C), extinction (cm.$^{-1}$), read at the 509mμ band against reagent blank, of the tris-(*o*-phenanthroline)–iron(II) complex obtained when the above sample is treated by Cameron's (1965) method and then diluted to 10ml. Concentrations of iron are calculated on the basis $\epsilon_{509\,m\mu} = 11{\cdot}0 \times 10^3\,M^{-1}cm.^{-1}$.

(A) mg. (±0·5%)	(B) mg. (±0·2%)	(C) Extinction	(D) % of Fe (dry basis)	(E) % of Fe (non-dry basis)
2·340	2·569	0·142	0·308	0·281
3·154	3·509	0·192	0·309	0·278
4·570	5·012	0·277	0·308	0·281
5·549	6·138	0·337	0·308	0·279
		Mean ...	0·308	0·280

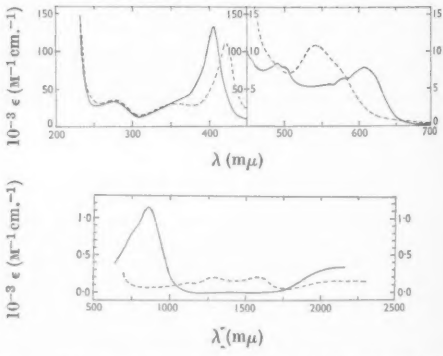

Fig. 2. Absorption spectra of sperm-whale ferrimyoglobin: ——, fluoride complex, Fe–F, at 25° and at pH7·0 in 1·5M-KF; – – – –, cyanide complex, Fe–CN, at 25° and at pH7·0 and I 0·05.

Table 2. *Characteristics of absorption spectra of sperm-whale ferrimyoglobin and derivatives at 25°*

max., Maximum at peak; min., minimum; sh., shoulder; ϵ (M^{-1} cm.$^{-1}$), extinction coefficient. Spectra are shown in Figs. 1 and 2.

Myoglobin derivatives									
$Fe^+(H_2O)$		Fe–OH		Fe–CN		Fe–F		Fe(II)–CO	
λ (mμ)	$10^{-3}\epsilon$	λ (mμ)	$10^{-3}\epsilon$	λ (mμ)	$10^{-3}\epsilon$	λ (mμ)	$10^{-3}\epsilon$	λ (mμ)	$10^{-3}\epsilon$
252 min.	23·0	264 min.	30·0	256 min.	29·9	252 min.	27·0	495–500 min.	5.20
280 max.	31·2	281 max.	32·1	280 max.	35·0	280 max.	33·5	540 max.	14·1
290 sh.	26·7	290 sh.	28·2	290 sh.	29·0	290 sh.	27·8	558 min.	10·1
311 min.	11·6	312 min.	14·0	310 min.	14·5	310 min.	12.5	567–569 sh.	11·3
409·5 max.	157	358 sh.	33·5	348 sh.	28·0	338–340 sh.	26·2	576 max.	12·3
460 min.	7·01	414 max.	97·2	359 max.	29·4	406 max.	133		
505 max.	9·47	515 min.	7·13	379 min.	26·1	470 min.	7·30		
578 min.	2·71	542 max.	9·48	423 max.	109·7	490 max.	8·31		
590 max.	2·76	565 min.	7·65	505 min.	6·99	500 sh.	7·80		
605 min.	2·71	582 max.	9·10	540 max.	10·7	540 min.	5·27		
635 max.	3·55	595 sh.	8·38	860–880 min.	0·069	560 sh.	5·63		
715 min.	0·150	675 min.	0·421	1080–1120 sh.	0·120	580 sh.	6·37		
1040 max.	0·893	740 sh.	0·554	1280 max.	0·202	609 max.	7·82		
1370 min.	0·025	825 max.	0·754	1420 min.	0·150	~760 sh.	0·820		
1510 max.	0·409	~1000 sh.	0·14	1580 max.	0·209	860 max.	1·15		
1660 min.	0·022	1365 min.	0·003	1750 min.	0·052	1160–1380 min.	0		
>2000 sh.	~0·60	1440 sh.	0·067	>2000 sh.	~0·16	>2000 sh.	~0·32		
		1520 max.	0·085						
		1660 min.	0						
		~1750 sh.	0·03						
		>2000 sh.	~0·20						

Table 3. *Comparison of the effects of temperature on the absorption spectra of sperm-whale and horse alkaline ferrimyoglobin*, Fe–OH

Changes in extinction/degree (ΔE/°c) are shown at two wavelengths, and are based on a solution having $E = 1{\cdot}000$ at the 542 mμ peak at 25°. Data for horse ferrimyoglobin are from George *et al.* (1961).

Wavelength (λ)	542 mμ	582 mμ
Sperm-whale ferrimyoglobin (range 4–39°)	2·4	1·8
Horse ferrimyoglobin (range 5–35°)	1·0	0·5

over, both compounds exhibit pH effects within the range pH 6–7; in the whale compounds this appears as a 2½% fall in absorption at the Soret peak, but in the horse compounds a haem-linked ionization could be inferred only indirectly from the variation of rate and equilibrium constants with pH (George & Hanania, 1955). A similar pH effect on the spectrum of Fe–F appears as a 10% change at the Soret peak in whale Fe–F and a smaller change in horse Fe–F. The spectra for both whale and horse $Fe^+(H_2O)$ and Fe–F are substantially unaffected by a temperature change from 4° to 40°.

With Fe–OH, the contrast between species is more marked, particularly in the visible region. Thus the characteristic double band in horse alkaline ferrimyoglobin (585 and 594 mμ) appears as a band and shoulder in whale alkaline ferrimyoglobin with about 7% stronger absorption in the latter. In this region the effect of temperature is also significant. The comparative data in Table 3 show that with sperm-whale Fe–OH the effect of temperature is more than twice as large as it is with horse Fe–OH. George, Beetlestone & Griffith (1961) have shown from a consideration of the magnetic and spectral properties of horse Fe–OH that the compound is an equilibrium mixture of 70% of high-spin and 30% of low-spin electronic states, and that the transition from high-spin to low-spin bonding is slightly exothermic. No corresponding magnetic data are available for whale Fe–OH, but since the effect of temperature on its spectrum is greater than for horse Fe–OH it may be concluded that a similar electronic transition in whale Fe–OH would be expected to be more exothermic.

Further information and details of spectral data for sperm-whale and horse myoglobins may be obtained from G. I. H. H. This investigation was supported by U.S. Public

Health Service Research Grant HE04994 and Research Fellowship HF11508 from the National Institutes of Health, Bethesda, Md., U.S.A., which we gratefully acknowledge.

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Isolation and Properties of a Basic Protein from Skeletal-Muscle Sarcoplasm

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(*Received* 18 *May* 1965)

1. An isolation procedure for preparing gram quantities of a previously undescribed protein from muscle is presented. 2. The protein amounts to over 2% of the sarcoplasmic proteins of pig longissimus dorsi muscle, but does not correspond to any glycolytic enzyme, nor to any of several other enzymes that have been tested for. 3. The protein is isoelectric between pH 8·5 and 9·0, has mol.wt. 34500 ± 500 and has $E^{1\%}_{281m\mu}$ 20·7. 4. The identities of nearly all the major protein bands obtained in electrophoresis on starch gel of pig longissimus dorsi muscle sarcoplasm have been established.

Much of the water-soluble protein in muscle consists of the 12 glycolytic enzymes, from phosphorylase to lactate dehydrogenase, together with creatine kinase and myoglobin. Czok & Bücher (1960) estimated that at least 70% of rabbit-muscle sarcoplasmic protein was accounted for by the known quantities of 15 enzymes, including the above; moreover, up to 40% as final yield could be obtained in crystalline forms. Nevertheless, some proteins have been isolated from the sarcoplasms of various species that have not been identified with known enzymes. Czok & Bücher (1960) obtained an unidentified nucleotide-containing protein from rabbit muscle, which co-crystallized with two of the glycolytic enzymes. Also, certain fish muscles contain large amounts of an unusual phenylalanine-rich protein (Jebsen & Hamoir, 1958; Henrotte, 1960). There must be many uncharacterized proteins in muscle sarcoplasm, and it appears that some of these can amount to a considerable proportion of the total protein; any enzymic functions that these may possess would be expected to be important ones in view of the quantities present.

Electrophoresis in starch gels demonstrates the variety of the proteins present in sarcoplasmic extracts (Giles, 1962; Hartshorne & Perry, 1962; Neelin, 1963; Scopes, 1964*a*). The main components usually number from 15 to 20, and these can be expected to correspond to the main enzymes mentioned above. A detailed investigation of the starch-gel electrophoretic pattern of pig longissimus dorsi muscle sarcoplasm resulted in identification of nearly all the major components (Scopes, 1964*b*). Only two proteins, which from their intensity of staining appeared to be present in a quantity of as much as 2% of the total, were not assigned an enzymic activity. One of these, the most basic of the major components, forms the subject of the present paper; a preliminary report on this basic protein has been presented (Scopes, 1964*c*).

The nomenclature system previously used for the starch-gel electrophoretic pattern of ox longissimus dorsi muscle sarcoplasm (Scopes, 1964*a*) divided the pattern into six areas, labelled A to F. Band F3 was indicated as a protein particularly labile in mildly acid conditions; the corresponding protein from pig muscle was rather more basic, and ran the farthest towards the cathode of all the pig proteins. Because of this protein's occurrence in the F-area of the gel in each species shown it has been called 'F-protein'. Details of the purification procedure from pig muscle and of some of the properties of F-protein are presented below; however, no physiological function of the protein has yet been ascertained.

METHODS

Starch-gel electrophoresis. An ice-cooled vertical apparatus, employing the discontinuous buffer system previously described (Scopes, 1964*a*), was used. The apparatus had a container attached to one side which was filled with ice–water. The water was directly in contact with the $\frac{1}{8}$in. Perspex plate on which the starch gel had been poured. The apparatus was set up in the cold room, and a potential difference of 400v could be applied (average 20v/cm.) without the temperature rising much above 5° in the gel. The current commenced close to 30mA (4·5mA/cm.^{2} gel), and fell to 18mA as the borate front spread through the inner gel. Electrophoresis was continued for 3·5hr., by which time the discontinuity had traversed 17cm. of the 18cm. inner gel. It was necessary to make up the latter with M-urea, which prevented deterioration at the low

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temperatures; also, its handling characteristics were improved.

Identification of protein bands. All glycolytic enzymes from phorphorylase to glyceraldehyde phosphate dehydrogenase, and also lactate dehydrogenase and some other dehydrogenases, were detected by incubation of a starch-gel strip with an NADH- or NADPH-producing assay medium, coupled to reduction of nitroblue tetrazolium in the usual fashion (Fine, Kaplan & Kuftinec, 1963). Other proteins were crystallized or highly purified, and their positions on the starch-gel pattern thus determined (Scopes, 1964*b*).

Estimations. Tyrosine and tryptophan in the purified protein were estimated spectrophotometrically by the method of Goodwin & Morton (1946). Tryptophan was chemically estimated by the method of Spies & Chambers (1948). Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951); routine measurements were made by reading the extinction at 280mμ, by using $E^{1\%}_{280m\mu}$ 10·3 for the (dialysed) whole sarcoplasm and $E^{1\%}_{280m\mu}$ 20·5 for the purified F-protein.

RESULTS

Isolation of F-protein

Muscle extract. Pig longissimus dorsi muscle, preferably from a 2–3-year-old animal, was excised shortly after death and cooled on ice. The pH of the muscle before homogenization was usually in the range 6·3–6·9. In one case the pH was 5·5 1hr. after death; this muscle showed the characteristics of 'watery pork' (Ludvigsen, 1954; Bendall & Lawrie, 1964), and the yield of F-protein was low, having been mostly denatured during the rapid post-mortem pH fall.

A 2kg. portion of muscle was chopped into small pieces and homogenized in 2vol. of cold iso-osmotic (3%, v/v) glycerol, containing EDTA (2mM), for 1min. in a Waring Blendor. The homogenate was adjusted to pH6·5 with M-tris or N-acetic acid and then centrifuged at 15000*g* for 10min. at 0°. The residue was discarded and the supernatant passed through glass wool to remove fat particles. The resultant fraction I had volume 4200ml. and contained 20–23mg. of protein/ml.

First acetone fractionation. Fraction I, pH6·5, was cooled to below 5° and 1800ml. of cold (−20°) acetone added quickly. The temperature of the mixture rose to about 12°; it was placed in a room at −10° and left to cool overnight. In the cold room (1°) a further 1650ml. of cold acetone was added over a period of 20min., while stirring, to make the total acetone concentration 45% (v/v). Stirring was continued for a further 20min. and then the mixture centrifuged in batches (5000*g* for 5min. at −7°). The residue contained about half the F-protein and over 90% of the other protein of the sarcoplasm. The supernatant was made 65% (v/v) with respect to acetone by quickly adding the requisite amount of cold acetone (approx. 1700ml.) and left to stand at −10°. After 1hr. the precipitated protein had sedimented and the bulk of the slightly cloudy supernatant could be decanted. The remaining slurry was centrifuged (10000*g* for 5min. at −10°) and all the supernatant discarded. This precipitate was dissolved in cold cacodylate buffer, pH6·8 (40mM-cacodylic acid–32mN-sodium hydroxide), and made to 500ml. with the buffer.

The 0–45% acetone precipitate was resuspended in 2000ml. of cold (−10°) 40% (v/v) acetone, containing cacodylic acid (20mM), and stirred vigorously for 30min. The mixture was centrifuged (5000*g* for 5min. at −7°) and the residue discarded. The supernatant was made 65% with respect to acetone, and the protein recovered as before and combined with the first 45–65% acetone fraction. The solution was centrifuged briefly at 0° to remove a small insoluble residue. The resultant fraction II had volume 1000ml. and contained 14mg. of protein/ml.

CM-Sephadex adsorption. Fraction II was passed through a CM-Sephadex C-50 column (2·5cm.× 10cm.), equilibrated with the cacodylate buffer, at 1°. The unadsorbed protein (fraction IIA) contained little or no F-protein and was discarded. The absorbed protein was desorbed with a single change to a borate buffer, pH8·5 (0·2M-boric acid–30mN-sodium hydroxide), and the eluant collected until dilution lowered the total extinction at 280mμ to 10. The resultant fraction III had volume 320ml. and contained 5mg. of protein/ml.

Second acetone fractionation. The pH of fraction III was lowered to 7·0 with M-cacodylic acid, and cold acetone added to give 30% (v/v) concentra-

Table 1. *Purification of F-protein from 2kg. of pig muscle*

Experimental details are given in the text.

Fraction	Volume (ml.)	Protein (mg.)	F-protein* (mg.)	Recovery* (%)	Purification*
I: pH6·5 iso-osmotic glycerol extract	4200	90000	2000	100	×1·0
II: combined 65% acetone ppts.	1000	14400	1400	70	×4·4
III: desorbed from CM-Sephadex	320	1600	1300	65	×36
IV: 50–62% acetone, passed through DEAE-cellulose	50	1050	1000	50	×43

* Estimated (see the text).

tion. The solution was left to stand at $-10°$ overnight. Further cold acetone was added slowly over a period of 20min. to make the total acetone concentration 50% (v/v). The mixture was centrifuged (5000*g* for 5min. at $-7°$), and the supernatant was made 62% (v/v) with respect to acetone and centrifuged at $-10°$. The latter precipitate was dissolved in the minimum amount of cold dilute tris buffer, pH 8·0 (2mN-acetic acid–3mM-tris), and this solution then passed through a small (1cm. × 2cm.) DEAE-cellulose column equilibrated with the same buffer. The unadsorbed protein was collected as the final preparation. The resultant fraction IV had volume 50ml. and contained 21mg. of protein/ml.

Fraction IV was dialysed at 0° against a pH 7·8 buffer (0·4M-boric acid–0·15M-tris–0·1mM-EDTA), in which it retained its properties for several weeks.

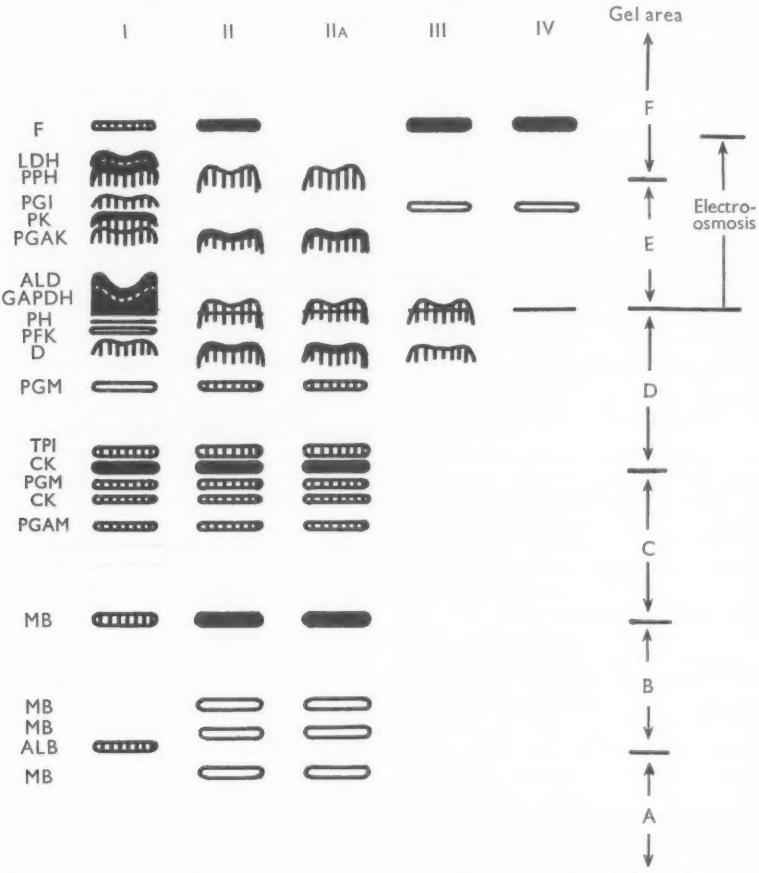

Fig. 1. Composition of protein fractions during preparation of F-protein: starch-gel electrophoretic patterns (only major bands shown). Experimental details are given in the text. On the left are given the identities of the bands. F, 'F-protein'; LDH, lactate dehydrogenase; PPH, phosphopyruvate hydratase (enolase); PGI, phosphoglucose isomerase; PK, pyruvate kinase; PGAK, phosphoglycerate kinase; ALD, aldolase; GAPDH, glyceraldehyde phosphate dehydrogenase; PH, phosphorylase; PFK, phosphofructokinase; D, 'D-protein'; PGM, phosphoglucomutase; TPI, triose phosphate isomerase; CK, creatine kinase; PGAM, phosphoglycerate mutase; MB, myoglobin; ALB, (serum) albumin. Fraction I, iso-osmotic glycerol–EDTA extract; fraction II, combined 65% acetone precipitates; fraction IIA, fraction II after passing through CM-Sephadex column; fraction III, desorbed from CM-Sephadex column; fraction IV, final preparation.

Since electrophoretic analysis was the only means of detecting F-protein, each fraction was submitted to starch-gel electrophoresis and the relative proportions and concentrations of the protein in each fraction were estimated in a semi-quantitative fashion. Recovery could be estimated by diluting the fractions to the equivalent volume of fraction I, and comparing the intensities of the F-protein bands after electrophoresis. The summary of the purification procedure given in Table 1 rests on the assumptions that in this particular preparation the recovery was 50% and that the final fraction was 96% pure. These values indicate that the F-protein constituted 2·2% of the sarcoplasmic protein; a lower recovery assumption would raise this percentage, and vice versa.

Fig. 1 shows the starch-gel electrophoretic patterns of the individual fractions. All major bands are shown, together with their identification. 'D-protein', except for its electrophoretic mobility, behaves similarly to Czok & Bücher's (1960) unidentified nucleotide-containing protein from rabbit, since it can be isolated by the same procedure.

No satisfactory conditions for crystallization of F-protein have been found. A few thin plate-like crystals were formed in an ammoniacal ammonium sulphate suspension, but the protein tended to coagulate rather than crystallize.

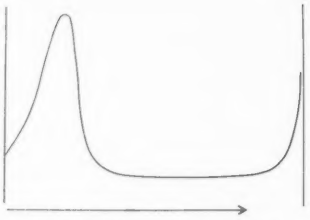

Fig. 2. Sedimentation diagram of F-protein. The conditions used were: 1% protein in 0·1 M-potassium phosphate buffer, pH 7·0; sedimentation for approx. 30 min. at 52640 rev./min.

Properties of F-protein

Behaviour in starch-gel electrophoresis, and purity of preparation. The protein exhibited two principal bands on the starch gel, and when freshly prepared the main one (F in Fig. 1) constituted about 90% of the total. The minor component (E) could not be separated from the major one by a variety of ion-exchange colums and other techniques. Moreover, on storage the F band became converted into component E, and after more prolonged storage (at 0° or −20°) the pattern split into several components in the E–F area. This deterioration was slowed in the presence of EDTA, but was accelerated by pH values above 8·5. Thus it appears that component E is a slightly modified form of F, perhaps a result of firm anion-binding during preparation and storage. To check that the multiplicity of bands was not due to polymerization, a well-aged preparation, which showed about five components on the starch gel, was subjected to an ultracentrifuge run. The sedimentation pattern (Fig. 2) was sufficiently symmetrical at all stages to rule out the possibility of polymerization. The sedimentation coefficient, S_{20}, calculated from this single run was 3·8 s.

The position that the F-protein ran to on the gel at pH 8·5 and 5° was slightly ahead of the position that a neutral substance was carried to by electro-osmosis. Thus it must have been negatively charged at this pH. But at pH 9·0 it ran behind the neutral point; also, it could be adsorbed on DEAE-cellulose at pH 9·0 but not at pH 8·5. From these observations it appears likely that the isoelectric point of F-protein is in the range 8·5–9·0.

The purity of the preparation, judged on the starch gel, was estimated to be about 95% (bands E and F combined). The protein was unstable in mildly acid conditions. This resulted in a low yield from 'watery pork' muscle (see the isolation procedure). At pH 4·50 and 30° it denatured rapidly, with first-order denaturation rate kinetics (k_1 0·14 min.$^{-1}$, by using the method described for the denaturation of creatine kinase; Scopes, 1965), until only 4% of the initial protein remained. This undenaturable protein corresponded to the small impurities detected on the starch gel, and so it was concluded that the preparation was 96% pure F-protein.

Ultraviolet spectrum. The protein contains an unusually high proportion of tryptophan, resulting in high absorption at about 280 mμ. The $\lambda_{max.}$ is 281 mμ, and $E^{1\%}_{281 m\mu}$ is 20·7, based on the freeze-dried dry weight. This value is considerably higher than for any other sarcoplasmic protein that has been reported. The u.v. spectrum in the range 200–320 mμ is shown in Fig. 3. Tryptophan 'shoulders' at 292 and 220 mμ are evident, and clearly no nucleotide is associated with the protein; the E_{281}/E_{260} ratio is 2·0.

Tyrosine and tryptophan contents were estimated as: tyrosine, $35·0 \pm 0·5$ residues/mol. of mol.wt. 10^5 (spectrophotometric); tryptophan $26·3 \pm 0·3$ residues/mol. of mol.wt. 10^5 (spectrophotometric) and $26·1 \pm 0·2$ residues/mol. of mol.wt. 10^5 (chemical).

Since there is good agreement between the chemical and spectrophotometric estimates of tryptophan, errors in the latter method due to buried or interacting residues exhibiting abnormal spectra are presumed insignificant.

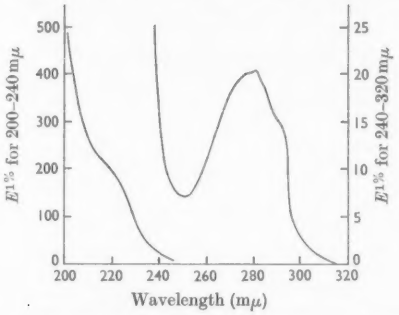

Fig. 3. Ultraviolet spectrum of F-protein in the ranges 200–240 mμ and 240–320 mμ. At $\lambda_{max.}$ 281 mμ, $E^{1\%}_{281 m\mu}$ is 20·7; at $\lambda_{min.}$ 250 mμ, $E^{1\%}_{250 m\mu}$ is 6·9.

Molecular weight. Molecular-weight determinations by osmotic pressure were carried out on solutions containing 4–16mg. of protein/ml. by using a simple osmometer (Adair, 1961). The extrapolated mol.wt. was 36000 ± 2000. By using this value, a more accurate estimate could be made from the tyrosine/tryptophan ratio of the protein (above). The ratio of these two amino acids is exactly 4:3, with 3 tryptophan residues/mol. representing a minimum mol.wt. of 11500 ± 150. Since the osmotic-pressure measurements gave a value of 36000 the molecule must contain 9 tryptophan residues and 12 tyrosine residues, and a more accurate value for the mol.wt. is 34500 ± 500.

Additional observations. (*a*) Amino acid analysis. A preliminary amino acid analysis revealed no marked abnormalities. The basic residues (excluding histidine) exceeded the acidic ones by 2–3/mol.; the histidine content was rather high (6% of hydrated amino acids).

(*b*) Partial specific volume. The density of a 2·35% solution was measured by using a pycnometer, and the partial specific volume calculated to be 0·732.

(*c*) Enzymic character. The protein's non-identity with any of the glycolytic enzymes is demonstrated in Fig. 1. In addition, the protein was assayed for the following activities: malate dehydrogenase, isocitrate dehydrogenase, β-hydroxybutyrate dehydrogenase, creatine kinase, adenosine triphosphatase, trypsin and several of the glycolytic enzymes. No enzymic activity was found.

DISCUSSION

No enzymic assay was available for F-protein, and analysis was only possible by using the electrophoretic technique. Nevertheless, it was soon clear in what ways it behaved differently to the other proteins in the sarcoplasm; its basic nature pointed the way to the main step in the purification procedure, with CM-Sephadex.

The protein amounts to over 2% of the sarcoplasmic protein in pig longissimus dorsi muscle. Similar proteins exist in ox and rabbit muscle (Scopes, 1964*a*), but they are present in slightly lower proportions in these species. Application of the isolation procedure described above to rabbit sarcoplasm was only partially successful, and the method would need some modification.

The possibility exists that F-protein could be an inactivated form, a sub-unit, or an isoenzyme of one of the major sarcoplasmic proteins for which no specific staining technique was available. This seems unlikely in view of its properties (in particular, the high tryptophan content), which significantly differ in detail from the main proteins that have been isolated.

Since several of the most important proteins in muscle, e.g. phosphoglucose isomerase and phosphoglycerate mutase, each amount to only about 1% of the total sarcoplasmic protein (Czok & Bücher, 1960), i.e. less than F-protein, then if the latter has any enzymic nature it may well be of fundamental importance to the normal functioning of the muscle.

I thank Dr G. S. Adair for carrying out the osmotic-pressure measurements, and also for help in some other determinations.

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Biochem. J. (1966) **98**, 198

Adenylosuccinate Synthetase and Adenylosuccinate Lyase from Plant Tissues

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(*Received* 30 *June* 1965)

1. Enzymes that convert IMP into adenylosuccinate (adenylosuccinate synthetase) and adenylosuccinate into AMP (adenylosuccinate lyase) were isolated from wheat germ and pea seeds and their properties are described. 2. These enzymes were purified approx. 200-fold from wheat-germ extracts. 3. A heat treatment provided adenylosuccinate lyase free of adenylosuccinate synthetase but the behaviour of the two enzymes was almost identical in a number of fractionation procedures. The two activities were finally separated by filtration on Sephadex G-100. 4. The identification of these enzymes in plant tissues is discussed in relation to the pathway of purine synthesis.

The pathway for synthesis of purine nucleotides in animal tissues and micro-organisms is well documented and many of the enzymes involved have been isolated and described (Buchanan, 1960; Schulman, 1961). Until recently, little information existed on purine synthesis in plant tissues. Studies on the incorporation of ^{14}C-labelled glycine and 5-aminoimidazole-4-carboxamide into purines of wheat leaves and embryos have now provided some evidence that a similar pathway is operative in plants (Robern, Wang & Waygood, 1965). There are also reports on the synthesis of glycinamide ribonucleotide in wheat-embryo extracts (Kapoor & Waygood, 1962) and the isolation of IMP dehydrogenase from pea seeds (Turner & King, 1961). The present paper describes the partial purification and properties of adenylosuccinate synthetase (IMP–L-aspartate ligase; EC 6.3.4.4) and adenylosuccinate lyase (adenylosuccinate AMP-lyase; EC 4.3.2.2) isolated from wheat germ. These enzymes catalyse the terminal steps for the new synthesis of AMP in liver and micro-organisms (Buchanan, 1960).

METHODS

Preparation of adenylosuccinate synthetase and adenylosuccinate lyase. A 30g. portion of an acetone-dried powder of wheat germ (Hatch & Stumpf, 1961) was shaken with 120ml. of 50mM-potassium phosphate buffer, pH7·5, for 30min. at room temperature. The suspension was pressed through fine muslin and the residual material re-extracted twice with 40ml. of the same buffer. These extracts were combined, cooled to 0° and centrifuged at 25000*g* for 20min. During the subsequent treatments the temperature was maintained between 0° and 5°. To 170ml. of the supernatant 15ml. of 5% (w/v) protamine sulphate was added slowly and the resulting precipitate was discarded. Small samples of the supernatant were dialysed for 16hr. against 10mM-tris–HCl buffer before being assayed for enzyme activity. The remaining supernatant was treated with saturated $(NH_4)_2SO_4$, pH7·0, and the fraction precipitating between 45% and 55% saturation was dissolved in 20ml. of 10mM-tris–HCl buffer, pH7·5, and dialysed against 3l. of the same buffer. This extract was adjusted to pH6·1 by the slow addition of 30mN-HCl and the precipitated protein was discarded. By further addition of HCl the pH was adjusted to 5·5 and the solution allowed to stand for 10min. at 5°. The precipitated protein was suspended in 10ml. of 10mM-potassium phosphate buffer, pH7·5. Undissolved protein was discarded and the supernatant was applied to a 12ml. column of calcium phosphate gel with a void volume of 8·5ml. After washing the column with 20ml. of 15mM-potassium phosphate buffer, pH7·5, the eluate from 16ml. of 40mM-potassium phosphate buffer, pH7·5, was collected. To reduce the volume of this fraction the protein was precipitated by the addition of solid $(NH_4)_2SO_4$ to give 65% saturation and then dissolved in 3ml. of 10mM-tris–HCl buffer, pH7·5, containing KCl (50mM). This fraction was applied to a Sephadex G-100 column 31cm. long × 5cm.^{2} cross-section and eluted with 10mM-tris–HCl buffer, pH7·5, containing KCl (25mM). Fractions of volume 5ml. were collected at a rate of 0·5ml./min. The protein content of each fraction was measured, and then 0·25ml. of serum albumin solution containing 40mg./ml. was added. Most enzyme studies were conducted with fractions obtained by Sephadex-gel filtration.

Enzyme assays. Adenylosuccinate synthetase was assayed essentially as described by Lieberman (1965). Reaction mixtures contained enzyme, tris–HCl buffer, pH8·3 (25μmoles), potassium phosphate, pH8·3 (10μmoles), $MgCl_2$ (5μmoles), tricyclohexylamine phosphoenolpyruvate (0·7μmole), pyruvate kinase (1·4units), L-aspartate (1·5μmoles), ATP (0·08μmole), GTP (0·08μmole) and IMP (0·5μmole) in a total volume of 1ml. Controls lacking IMP were run for every reaction. Reactions were stopped with

0·7ml. of 9% (v/v) $HClO_4$, and IMP was added to the control systems. After centrifuging to remove precipitated protein the increase in extinction at 275mμ, compared with controls, was measured. The amount of IMP converted into adenylosuccinate was calculated from the published spectra of IMP (Beaven, Holiday & Johnson, 1955) and adenylosuccinate (Carter & Cohen, 1956). By assaying adenylosuccinate synthetase at pH 8·3 the proportion of the product that underwent further breakdown to AMP remained small with most enzyme fractions. The amount of AMP formed could be calculated from the ratio of the observed extinction changes at 280mμ and 270mμ. For some studies L-[U-^{14}C]aspartate was used. In this case reactions were stopped by heating at 100° for 2min., and then samples were chromatographed on Whatman no. 1 paper with propyl acetate–90% (w/v) formic acid–water (11:5:3, by vol.) (Bieleski & Young, 1963) as the developing solvent. Activity was determined by measuring the areas of the peaks traced with a recording strip counter. When necessary these results were confirmed by counting the areas containing aspartate and adenylosuccinate with a Geiger–Müller tube.

Adenylosuccinate lyase was generally assayed by measuring the conversion of AMP into adenylosuccinate in the presence of an excess of fumarate (Carter & Cohen, 1956). Reaction mixtures contained enzyme, potassium phosphate buffer, pH 6·6 (50μmoles), fumarate (6μmoles) and AMP (0·6μmole) in a total volume of 1ml. The subsequent procedure including the use of controls was the same as that described for adenylosuccinate synthetase except that activity was measured by the change in extinction at 280mμ. For some studies the conversion of adenylo[^{14}C]-succinate into radioactive fumarate was measured in reaction mixtures containing 40mM-potassium phosphate buffer, pH 6·6, and enzyme in a total volume of 0·2ml. Activity was measured by the procedure described for the radiotracer studies with adenylosuccinate synthetase.

Fumarate-hydratase activity was measured in reaction mixtures of the same composition as that described for adenylosuccinate lyase except that AMP was omitted. The decrease in extinction at 275mμ was used as a measure of enzyme activity. The product, malate, was identified by its chromatographic mobility with the three solvents and the detection procedure described by Hatch & Stumpf (1961).

A unit of adenylosuccinate-synthetase or adenylosuccinate-lyase activity is defined as the amount of enzyme required to utilize 1μmole of substrate/min. under the conditions of assay. All assays were conducted at 30°.

Determination of protein. The protein content of enzyme preparations was determined by the method of Warburg & Christian (1941).

MATERIALS

The following compounds were obtained from commercial sources: ATP, GTP, ADP, IDP, AMP, IMP, CMP, adenosine, inosine, adenine, tricyclohexylamine phosphoenolpyruvate, pyruvate kinase and protamine sulphate from Sigma Chemical Co. (St Louis, Mo., U.S.A.); L-[U-^{14}C]aspartic acid (6·1mc/m-mole) from The Radiochemical Centre (Amersham, Bucks.); calcium phosphate gel (hypatite C) from Clarkson Chemical Co. (Williamsport, Pa., U.S.A.); Sephadex G-100 from Pharmacia (Uppsala, Sweden).

Adenylo[^{14}C]succinate was prepared by incubating adenylosuccinate synthetase (0·01 unit) in a 3ml. reaction mixture of the composition described above except that non-radioactive aspartate was replaced by 1·5μmoles of [U-^{14}C]aspartate ($2·1 \times 10^7$ disintegrations/min.). The reaction mixture was incubated at 30° for 2hr., heated at 100° for 2min. and then chromatographed as a band on a large sheet of Whatman no. 3MM paper with propyl acetate–90% (v/v) formic acid–water (6:3:1, by vol.) as the developing solvent. The adenylosuccinate band was located by radioautography and eluted from the paper with 5mM-potassium phosphate buffer, pH 7·0. About 40% of the radioactivity supplied was recovered as adenylosuccinate. Incubation of the product with adenylosuccinate lyase provided evidence for its identity and purity with respect to radioactivity. When low concentrations (0·037mM) were supplied all the radioactivity was recovered in fumaric acid.

Table 1. *Purification of adenylosuccinate synthetase and adenylosuccinate lyase*

Reaction mixtures contained approx. 1×10^{-3} unit of adenylosuccinate synthetase and approx. $1·5 \times 10^{-3}$ units of adenylosuccinate lyase. The remaining components of the reaction mixtures and the assay methods are described in the Methods section. The incubation time was 60min.

		Adenylosuccinate synthetase		Adenylosuccinate lyase	
Enzyme fraction	Concn. of protein (mg./ml.)	Total units	Specific activity (unit/mg. of protein)	Total units	Specific activity (unit/mg. of protein)
25000*g* supernatant	22	—	—	3·9	0·010
Protamine sulphate supernatant	12	0·68	0·003	4·0	0·017
45–55% satd. $(NH_4)_2SO_4$	19	0·54	0·012	3·1	0·07
Ppt. between pH 6·2 and pH 5·5	10·6	0·31	0·03	2·0	0·18
Calcium phosphate-gel fraction	1·4	0·27	0·12	1·8	0·80
Sephadex fraction 11	0·58	0	0	0·52	1·8
Sephadex fraction 12	0·32	0·038	0·24	0·45	2·9
Sephadex fraction 14	0·30	0·082	0·55	0	0

RESULTS

Purification of adenylosuccinate synthetase and adenylosuccinate lyase. Data on the purification are given in Table 1. The specific activities of both enzymes in selected fractions from Sephadex G-100 were more than 200 times those in extracts obtained after treatment with protamine sulphate. The protamine sulphate treatment was included primarily to remove nucleic acids. With the assay procedure employed no adenylosuccinate-synthetase activity was detected in the 25000*g* supernatant although it was possible to detect activity after treatment with protamine sulphate. An extract of the protamine sulphate precipitate, obtained as described by Mendicino (1960), showed neither adenylosuccinate-synthetase nor adenylosuccinate-lyase activity.

Separation of the two enzymes proved difficult, their behaviour during fractionation by ammonium sulphate and isoelectric precipitation and chromatography on calcium phosphate being almost identical (Table 1). Chromatography at pH 7·4 on DEAE-cellulose was also unsuccessful. Procedures for the quantitative assay of adenylosuccinate synthetase in extracts containing adenylosuccinate lyase are described in the Methods section. The assay of adenylosuccinate lyase in the presence of fumarate hydratase also provided a potential problem. However, under the conditions employed for the assay only a small fraction of the fumarate supplied was converted into malate. Filtration on Sephadex G-100 provided adenylosuccinate synthetase free of adenylosuccinate lyase and adenylosuccinate lyase free of fumarate hydratase (Fig. 1). Fumarate hydratase moved with the protein fraction that was completely excluded from the gel.

Characterization of reactions. The observed difference spectra for the reactions catalysed by adenylosuccinate synthetase and adenylosuccinate lyase were in close agreement with those calculated from the published spectra of IMP, AMP and adenylosuccinate. For accurate determination of extinction differences below 270mμ further dilution of the perchloric acid extracts of reaction mixtures was necessary owing to the high blank extinction in this region.

The product formed from IMP and L-[U-^{14}C]-aspartate in the presence of adenylosuccinate synthetase was converted exclusively into radioactive fumarate when incubated with a purified preparation of adenylosuccinate lyase. The latter product co-chromatographed on paper with unlabelled fumarate in three different solvents (Hatch & Stumpf, 1961).

Properties of adenylosuccinate synthetase. The reaction rate was constant for at least 90min. under the conditions of assay (Table 2). Separate studies with 50mM-potassium phosphate buffer showed the optimum pH for activity was between

Table 2. *Properties of adenylosuccinate synthetase*

The composition of the complete assay system, buffered to pH 8·3 with 25mM-tris–HCl, is described in the Methods section. Buffers described in the Table replaced the above buffer. Each reaction mixture contained an equal sample of fraction 14 from Sephadex G-100 (see Fig. 1), containing 1×10^{-3} unit.

Modification of complete assay system	Reaction time (min.)	Adenylosuccinate-synthetase activity (mμmoles/reaction mixture)
None	30	27
None	60	55
None	90	80
None	120	103
50mM-Glycine–KOH buffer, pH 8·3	60	54
75mM-Tris–HCl buffer, pH 8·3	60	39
25mM-Tris–HCl buffer, pH 7·4	60	63
25mM-Tris–HCl buffer, pH 9·4	60	20
50mM-Potassium phosphate buffer, pH 7·6	60	70
With 3mM-aspartate	60	57
With 1·5mM-asparagine; aspartate omitted	60	0
With 1·5mM-glutamate; aspartate omitted	60	3
With 0·5mM-inosine; IMP omitted	60	0
With 0·5mM-IDP; IMP omitted	60	4
With 0·2mM-GTP; phosphoenolpyruvate omitted	60	37
With 0·2mM-GTP; phosphoenolpyruvate and $MgCl_2$ omitted	60	0
GTP omitted	60	0

7·5 and 7·8. However, 25mM-tris–hydrochloric acid buffer, pH 8·3, was used as a routine to minimize the conversion of adenylosuccinate into AMP when adenylosuccinate lyase was present. At this concentration tris–hydrochloric acid buffer did not inhibit the enzyme compared with 50mM-glycine–potassium hydroxide buffer at the same pH. With 75mM-tris–hydrochloric acid buffer a 30% inhibition was observed.

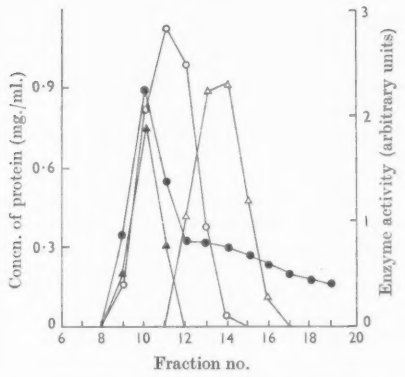

Fig. 1. Separation of adenylosuccinate synthetase (△), adenylosuccinate lyase (○) and fumarate hydratase (▲) on Sephadex G-100; the distribution of protein (●) is also shown. The column volume was 155ml., the void volume was 45ml. and the volume of each fraction was 5ml. Other details are described in the Methods section. Enzyme activity is expressed in arbitrary units to bring the activity/unit fraction volume to a similar value for each enzyme.

Studies of substrate requirements for adenylosuccinate synthetase are recorded in Table 2. Aspartate was essential for activity. The enzyme was apparently saturated with 1·5mM-aspartate, 3mM-aspartate giving no significant increase in activity. D-Aspartate (in a separate experiment) and asparagine could not replace L-aspartate, and the activity observed with glutamate was barely significant. No activity was observed when IMP was replaced by inosine, and the low activity observed with IDP may have been due to contaminating traces of IMP. The pyruvate-kinase system for generating GTP was apparently essential for maintaining maximum activity since even with 0·2mM-GTP activity was decreased when phosphoenolpyruvate was omitted. Lieberman (1956) reported similar observations. ATP could not replace GTP since no activity was observed when the latter was omitted.

Properties of adenylosuccinate lyase. Under the conditions of assay the rate of the reaction catalysed by adenylosuccinate lyase remained constant for at least 60min. (Table 3). At 60min. 16% of the AMP was utilized and according to the data of Carter & Cohen (1956) approx. 50% would be converted into adenylosuccinate at equilibrium with the concentrations of substrates used. Optimum activity was observed between pH 6·5 and 7·2 and about one-third of maximum activity at pH 5·6 and pH 8·3. Fumarate was essential for activity and increasing its concentration from 6mM to 12mM increased the rate by only 5%. There was no

Table 3. *Properties of adenylosuccinate lyase*

The composition of the complete assay system, buffered to pH 6·6 with 50mM-potassium phosphate, is described in the Methods section. Buffers described in the Table replaced the above buffer. An equal sample of fraction 12 from Sephadex G-100 (see Fig. 1), containing $1·5 \times 10^{-3}$ units of activity, was supplied to each reaction mixture.

Modifications of complete assay system	Reaction time (min.)	Adenylosuccinate-lyase activity (mμmoles/reaction mixture)
None	20	34
None	40	69
None	60	101
None	80	129
50mM-Potassium phosphate buffer, pH 5·6	60	36
50mM-Potassium phosphate buffer, pH 7·2	60	102
50mM-Potassium glycinate buffer, pH 8·3	60	33
25mM-Tris–HCl buffer, pH 8·3	60	31
Fumarate omitted	60	0
With 12mM-fumarate	60	106
With 1mM-AMP	60	99
With 0·6mM-CMP; AMP omitted	60	0
With 0·6mM-adenosine; AMP omitted	60	0
With 0·6mM-adenine; AMP omitted	60	0

increase in activity when the concentration of AMP was increased to 1mM and no change in extinction was observed when AMP was replaced by CMP, adenosine or adenine.

Michaelis constants. Michaelis constants were calculated from the reciprocals of activity and substrate concentration (Lineweaver & Burk, 1934). The necessity for measuring initial rates with subsaturating concentrations of substrate presented some limitations to accuracy. Calculations were made from extinction values between 0·2 and 0·04. By using the spectrophotometric method the K_m values for IMP and aspartate for the reaction catalysed by adenylosuccinate synthetase were 0·09mM and 0·43mM respectively. With the radiotracer method K_m for aspartate was 0·38mM.

For the reaction catalysed by adenylosuccinate lyase the K_m values for adenylosuccinate, AMP and fumarate were 0·03mM, 0·11mM and 0·65mM respectively. The radiotracer method was used to determine K_m for adenylosuccinate.

Enzyme stability. Heating the enzyme fraction obtained by calcium phosphate-gel chromatography at 60° for 5min. completely inactivated adenylosuccinate synthetase but only 25% of the adenylosuccinate-lyase activity was lost. Both enzymes retained activity when held at pH 5·2 and 5° for 10min. The enzyme activity in all fractions except those obtained by Sephadex-gel filtration was stable for at least 3 weeks when stored at −15°. After 3 weeks at −15° the adenylosuccinate synthetase of fraction 14 and the adenylosuccinate lyase of fraction 12 from Sephadex G-100 lost 15 and 25% respectively of their original activities.

Occurrence in other plant tissues. Adenylosuccinate synthetase and adenylosuccinate lyase were present in a 45–55% saturated ammonium sulphate fraction from pea seeds prepared as described by Hatch & Turner (1960). Failure to detect these enzymes in a 40–60% saturated ammonium sulphate fraction obtained from extracts of immature leaf and stem from sugar-cane, shoots from sugar-cane sets and potato leaves may have been due to the presence of interfering enzymes. All these extracts contained high nucleoside monophosphate-ribohydrolase activity but this enzyme was not detectable in wheat and pea preparations.

DISCUSSION

In most essential respects the properties of the adenylosuccinate synthetase and adenylosuccinate lyase from wheat germ were similar to those for the same enzymes isolated from animal tissue and micro-organisms (Carter & Cohen, 1956; Lieberman, 1956; Miller, Lukens & Buchanan, 1959). A marked difference was observed between the wheat-germ adenylosuccinate synthetase and the bacterial enzyme (Lieberman, 1956) and tumour enzyme (Atkinson, Morton & Murray, 1964) with respect to solubility at low pH. The latter enzymes remained soluble at pH 5·4 and this step was used to remove adenylosuccinate lyase. In the wheat-germ extracts both adenylosuccinate synthetase and adenylosuccinate lyase were completely precipitated as the pH was lowered from 6·2 to 5·5. It is of practical interest that the yeast adenylosuccinate lyase was freed of fumarate hydratase by heating preparations at 60° for approx. 10min. (Carter & Cohen, 1956). Under these conditions wheat-germ fumarate hydratase lost no activity but almost 50% of the adenylosuccinate-lyase activity was destroyed.

The studies described in the present paper provide further support for the conclusion of Robern *et al.* (1965) that the pathway for the new synthesis of purine nucleotides in plants is similar to that operative in animal tissues and micro-organisms, the terminal steps for AMP synthesis being catalysed by adenylosuccinate synthetase and adenylosuccinate lyase. There is now strong evidence that the conversion of adenylosuccinate into AMP and the cleavage of 5-aminoimidazole-4-*N*-succinocarboxamide ribotide, an earlier step in purine synthesis, are catalysed by the same protein entity in liver, moulds and micro-organisms (Flaks & Lukens, 1963). A similar double function for the plant enzyme appears likely.

The demonstration of adenylosuccinate synthetase in plants is of interest with respect to the possible mode of action of hadacidin (*N*-formylhydroxyaminoacetic acid) as an inhibitor of growth of sugar-cane (P. E. Robinson, personal communication) and many other plants (Gray, Gauger, Dulaney, Kaczka & Woodruff, 1964). Growth-inhibitory effects of hadacidin on tumour cells (Kaczka, Gitterman, Dulaney & Folkers, 1962) and bacteria (Shigeura & Gordon, 1962*a*) has been attributed entirely to inhibition of adenylosuccinate synthetase (Shigeura & Gordon, 1962*b*,*c*).

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The Purification and Properties of a Penicillinase whose Synthesis is Mediated by an R-Factor in *Escherichia coli*

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(*Received* 14 *June* 1965)

1. The penicillinase (β-lactamase) from *Escherichia coli* strain TEM has been purified and its activity against a range of penicillin and cephalosporin derivatives measured. 2. The enzyme shows little resemblance to penicillinases from *Bacillus cereus*, *Bacillus licheniformis* and *Staphylococcus aureus*. 3. The molecular weight of the enzyme is $16700 \pm 5\%$, which is about half the value obtained for other penicillinases. 4. The enzyme is most similar in properties to a crude preparation of a penicillinase from *Klebsiella* (*Aerobacter*) *aerogenes*, but clearly different from crude enzyme preparations from other strains of *E. coli*. 5. Since penicillinase synthesis in *E. coli* strain TEM is mediated by an R-factor known to infect many other species of Enterobacteriaceae, the appearance of similar enzymes in other Gram-negative species is not surprising.

A number of penicillinases (penicillin β-lactamases; EC 3.5.2.6) from Gram-positive species [i.e. *Bacillus cereus* (Kogut, Pollock & Tridgell, 1956; Pollock, Torriani & Tridgell, 1956), *Bacillus licheniformis* (Pollock, 1965*a*) and *Staphylococcus aureus* (Richmond, 1963, 1965)] have been purified and their properties examined, but hitherto no penicillinase from Gram-negative species has been purified to any extent. The isolation by Kontomichalou (Datta & Kontomichalou, 1965) of a strain of *Escherichia coli* (strain TEM) that synthesized relatively large amounts of penicillinase was a convenient opportunity to attempt the isolation of a penicillinase from a Gram-negative species. The strain has the added interest that the genome responsible for penicillinase synthesis is carried on an extrachromosomal R-factor (Datta & Kontomichalou, 1965). The enzyme should not therefore be regarded strictly as 'the penicillinase of *E. coli*', but rather as the penicillinase synthesized by *E. coli* when it is carrying this particular R-factor. It is known that R-factors, including that present in strain TEM, can be transferred to a wide range of Enterobacteriaceae, but whether the properties of the enzyme are modified by the transfer of the gene to a new host is unknown.

The purified enzyme shows a number of differences from penicillinases obtained from Gram-positive bacteria; perhaps the most striking is the markedly lower molecular weight (approx. 16700 as against about 30000 for the penicillinases from the Gram-positive species). In addition, enzyme synthesis in *E. coli* strain TEM is constitutive, which is unlike the situation found with all strains of Gram-positive bacteria that synthesize penicillinase except *B. cereus* strain 5 (Sneath, 1955).

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METHODS AND MATERIALS

Organism and medium. The purification has been developed with *E. coli* strain TEM, a strain in which the penicillinase gene is carried on an R-factor (Datta & Kontomichalou, 1965). This strain is convenient since it synthesizes a relatively large amount of penicillinase (about 80 enzyme units/mg. dry wt. of organisms). The organism was grown in 1% CY medium (Novick, 1962*a*), which has the following composition: sodium β-glycerophosphate, 0·12M; $MgSO_4$, 1mM; trace-metal solution, 0·02ml./l.; yeast extract (Difco), 1·0% (w/v); acid-hydrolysed casein (Difco), 1·0% (w/v); glucose, 0·4%. The trace-metal solution contained: $CuSO_4,5H_2O$, 0·5%; $ZnSO_4,7H_2O$, 0·5%; $FeSO_4,7H_2O$, 0·5%; $MnCl_2,4H_2O$, 0·2%; conc. HCl, 10% (v/v). The glucose and β-glycerophosphate were autoclaved separately and added immediately before use. Agar (2·0%, w/v) was added to 1% CY medium when required.

Column materials. DEAE-cellulose powder (formerly known as grade DE50, now as DE11) was obtained from Whatman Products, Reeve Angell, London. Sephadex G-75 (bead form) and Sephadex G-50 (medium grade) were obtained from Pharmacia, Uppsala, Sweden.

Growth of organisms. The R-factor is lost from *E. coli* strain TEM at a frequency of about 1/1000 divisions, and this strain was therefore maintained on 1% CY agar plates

containing 25μg. of ampicillin/ml. For routine growth purposes a colony was picked from the ampicillin–agar plate and grown overnight in 100ml. of 1% CY medium on a shaker at 35°. In the morning this culture was diluted to a density of about 0·2mg. dry wt. of organisms/ml. in fresh 1% CY medium and shaken at 35° until the culture density reached 2–3mg. dry wt. of organisms/ml. At this point the organisms were collected by centrifugation and used for stage 1 of the purification procedure (see the Results section). For large-scale preparations the cultures were shaken at 35° in 5l. conical flasks containing not more than 1100ml. of medium.

Enzyme assays. Penicillinase activities are expressed in the units defined by Pollock & Torriani (1953) for *B. cereus* penicillinase: 1 unit≡1μmole of benzylpenicillin hydrolysed/hr. at 30° and pH7·0. Penicillinase was assayed iodometrically at pH7·0 by the method of Perret (1954) as modified by Novick (1962*a*). The hydrolysis of cephalosporin derivatives was measured similarly except that the amount of cephalosporin destroyed was calculated on a basis of 1mole of hydrolysed cephalosporin≡4 equiv. of iodine (Alicino, 1961). Michaelis constants were determined by the microiodometric assay (Novick, 1962*b*).

Preparation of chromatography and Sephadex columns. DEAE-cellulose was suspended in 0·1M-Na_2HPO_4–KH_2PO_4 buffer, pH7·0, and the fines were removed. The column was then poured in 0·1M-phosphate buffer, pH7·0, and washed with this buffer until the effluent had reached pH7·0. The column was prepared for use by washing with about 1l. of water. The DEAE-cellulose columns used in these experiments were about 1·5cm. diam. × 15–20cm. long, and were used at 2°.

The Sephadex columns (approx. 2cm. × approx. 140cm.) were prepared as described in the booklet issued by the makers. The columns were poured in water and then equilibrated against 0·1M-Na_2HPO_4–KH_2PO_4 buffer, pH7·0, before use. They were used at room temperature. In between periods of use the columns were either equilibrated against 0·1N-acetic acid or 0·1M-phosphate buffer saturated with $CHCl_3$.

Protein estimations. Protein was normally estimated by the Lowry method (Lowry, Rosebrough, Farr & Randall, 1951), with an aqueous solution of bovine serum albumin as standard. Elution of proteins from columns was followed by the extinction at 280mμ.

Starch-gel electrophoresis. Separation of proteins by starch-gel electrophoresis was carried out at pH8·0 in the discontinuous buffer systems described by Poulik (1957).

RESULTS

Location and liberation of penicillinase in E. coli *strain TEM*

As with other species of Gram-negative bacilli that produce penicillinase, the enzyme of *E. coli* strain TEM is associated with the cells, and normally none is found in the culture supernatant. The enzyme in the intact organisms does not appear to be readily accessible to substrate, since a 130-fold increase in enzyme activity occurs on ultrasonic disruption under the conditions described below. Other treatments designed to disrupt the bacterial surface, such as toluene, EDTA at pH8·0 (Repaske, 1958) or EDTA plus 20% (w/v) sucrose at pH8·0 (Neu & Heppel, 1964), liberated up to 35% of the total enzyme in a soluble form. The fact that the enzyme is liberated from the bacteria under conditions in which the cytoplasmic membrane is protected (Neu & Heppel, 1964) suggests that penicillinase may be one of the 'periplasmic enzymes' of *E. coli*.

Since the most efficient liberation of enzyme was achieved by ultrasonic disintegration, this was the method chosen as a preliminary step in purification of the enzyme.

Purification of the penicillinase

The recoveries at each stage are shown in Table 1. All phosphate buffers were made from disodium hydrogen phosphate and potassium dihydrogen phosphate.

Stage 1. Organisms from the late exponential phase of growth were harvested by centrifugation, washed well in 0·1M-phosphate buffer, pH7·0, and resuspended in a similar buffer to a density of about 100mg. dry wt. of organisms/ml. The

Table 1. *Summary of the purification of penicillinase from* E. coli

Experimental details are given in the text. The starting material was 3l. of culture containing 11·1g. dry wt. of organisms.

Stage	Procedure	Enzyme activity recovered (units)	Specific enzyme activity (units/μg. of enzyme)	Recovery (%)	
				Per stage	Overall
1	Ultrasonic disintegration	683000	1	100	100
2	Centrifugation and dialysis	674000	0·82	98·6	98·6
3	Adsorption and elution on DEAE-cellulose	491800	14·7	72·9	72·0
4	Chromatography on DEAE-cellulose	298200	41·2	60·6	43·6
5	Sephadex G-50	261000	68·3	87·6	38·2
6	Sephadex G-75	234000	81·2	89·6	34·2
7	Rechromatography on DEAE-cellulose	147400	80·7	62·9	21·6

suspension was stored at 2° and 8·0ml. portions were broken for 2min. at 2° in a Mullard 25kcyc./sec. ultrasonic disintegrator. The temperature was kept low by packing the transducer cup in a vacuum flask filled with crushed ice. The broken cells were stored at 2° until all the suspension had been broken and then centrifuged at 5000*g* for 20min. at 2° to remove whole cells and cell debris.

Stage 2. The supernatant from the low-speed centrifugation was centrifuged again (first at 30000*g* for 4hr. at 2°, then at 105000*g* for 2hr. at 2°) and the pellets were discarded. The supernatant from these centrifugation steps was then dialysed twice for 16hr. each at 2° against 0·01M-phosphate buffer, pH7·0. During this step a slight precipitate formed, which was removed by centrifuging at 5000*g* for 15min. at 2°.

Stage 3. The enzyme solution, after dialysis, was loaded on to a DEAE-cellulose column (prepared as described in the Methods and Materials section) and washed on the column with about 500ml. of water. A great deal of protein and some pigmented material passed straight through the column at this stage and these were discarded. The enzyme was then eluted (rate of flow, 1·0ml./min.) with 200ml. of 0·03M-phosphate buffer, pH7·0, and 6ml. fractions were collected. The fractions containing the enzyme were combined, freeze-dried, dissolved in about 5·0ml. of water and dialysed overnight against 0·01M-phosphate buffer, pH7·0, at 2°.

Stage 4*: chromatography on DEAE-cellulose.* The enzyme preparation was reloaded on to a DEAE-cellulose column prepared as described in the Methods and Materials section and washed with 200ml. of water. The enzyme was then eluted (6ml. fractions) with a concentration gradient of phosphate buffer, pH7·0. In an experiment in which about 600000 units of enzyme were loaded on to a column 1·2cm. diam. × 20cm. long, the gradient was constructed with 300ml. each of 0·003M- and 0·05M-phosphate buffer. Fig. 1 shows a typical elution pattern. The fractions containing the enzyme (20–30, Fig. 1; total vol. 66ml.) were pooled, freeze-dried, dissolved in about 2·0ml. of water and dialysed overnight against 0·1M-phosphate buffer, pH7·0, at 2°.

Stage 5*: separation through Sephadex G-50.* The enzyme was loaded on to a Sephadex G-50 column (2·1cm. × 138cm.) that had already been equilibrated against 0·1M-phosphate buffer, pH7·0, and eluted (5·8ml. fractions) with similar buffer. Fig. 2 shows the elution diagram obtained. Fractions containing penicillinase at a specific activity greater than 65units/μg. of protein were pooled, freeze-dried, dissolved in 2·0ml. of water and dialysed overnight against 0·1M-phosphate buffer, pH7·0, at 2°.

Stage 6*: separation through Sephadex G-75.* The enzyme was loaded on to a Sephadex G-75 column (1·9cm. × 157cm.) that had already been equilibrated against 0·1M-phosphate buffer, pH7·0, and the enzyme eluted with similar buffer. Fig. 3 shows the elution pattern obtained at this stage. About 85% of the total enzyme activity was obtained in three 6ml. fractions with specific enzyme activities of 79·6, 81·2 and 80·3units/μg. of protein, and this suggests that the enzyme is nearly pure. The purity of the enzyme was checked by electrophoresis in starch gel (see the Methods and Materials section), where only one protein band which corresponded with enzyme activity could be detected. Examination of the purified enzyme in the

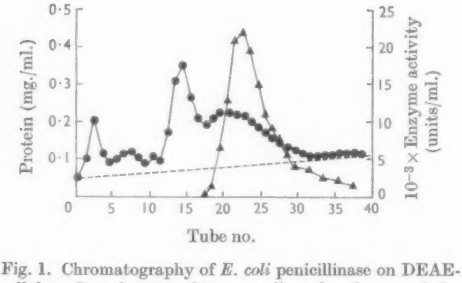

Fig. 1. Chromatography of *E. coli* penicillinase on DEAE-cellulose. Samples (6·0ml.) were collected and assayed for penicillinase (▲) and protein (●) content. The broken line indicates the non-specific reaction obtained in a dummy run with a column of similar dimensions. Protein estimations were carried out by the Lowry method (Lowry *et al.* 1951).

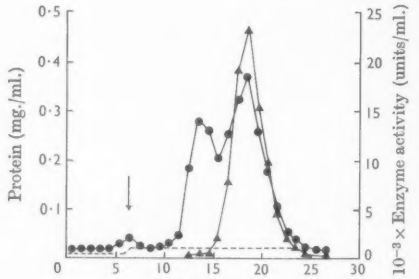

Fig. 2. Separation of *E. coli* penicillinase through Sephadex G-50 equilibrated against 0·1M-Na_2HPO_4–KH_2PO_4 buffer, pH7·0. Each fraction (5·8ml.) was assayed for protein (●) and enzyme (▲) content. The broken line indicates non-specific reaction. The arrow indicates the exclusion volume of the column. About 15 fractions were discarded at the beginning of the run. Protein estimations were carried out by the Lowry method (Lowry *et al.* 1951).

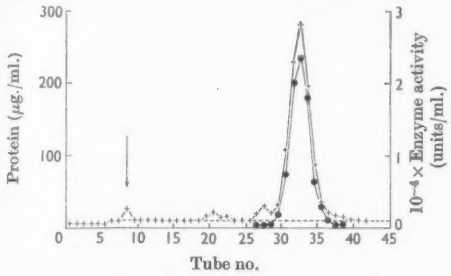

Fig. 3. Separation of *E. coli* penicillinase through Sephadex G-75 equilibrated against 0·1M-Na_2HPO_4–KH_2PO_4 buffer, pH 7·0. Each fraction (6·0 ml.) was assayed for protein (+) and enzyme (●) content. The broken line indicates non-specific reaction. The arrow marks the exclusion volume of the column. About 15 fractions were discarded at the beginning of the run. Protein estimations were carried out by the Lowry method (Lowry *et al.* 1951).

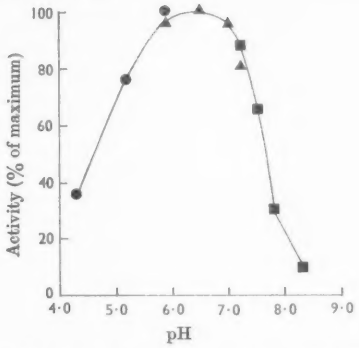

Fig. 4. pH–activity curve for the purified penicillinase from *E. coli* strain TEM. Sodium acetate buffers (0·1M) were used for the range pH 4·3–5·9 (●), Na_2HPO_4–KH_2PO_4 buffers (0·1M) for the range pH 5·9–7·2 (▲) and tris–HCl buffers (0·1M) for the range pH 7·2–8·3 (■).

analytical ultracentrifuge (enzyme concn., 8·8 mg./ml. in 0·03M-phosphate buffer, pH 7·0) showed a single symmetrical peak.

Stage 7: rechromatography on DEAE-cellulose. To check the purity of the enzyme the preparation was chromatographed once more on DEAE-cellulose, as described in stage 4. The protein peak coincided with the peak in enzyme activity exactly, and the specific activity of the enzyme across the peak was nearly constant (min. 76·4, max. 82·1 units/μg. of protein).

Properties of the penicillinase

Molecular weight. The molecular weight of the enzyme was found by the equilibrium-ultracentrifugation method of Yphantis (1964) to be 16700 ± 5%. This value agrees well with the fact that the enzyme was almost completely excluded by Sephadex G-50. The enzyme had $S_{20,w}$ 1·85s when compared with lysozyme by centrifugation in a sucrose density gradient (Charlwood, 1964).

pH–*activity curve.* The pH–activity curve (Fig. 4) was determined over the range pH 4·3–8·3 in various buffer mixtures. The enzyme has a broad optimum over the range pH 5·8–7·0. As with the penicillinase of *S. aureus* (Richmond, 1963), the enzyme is remarkable for a sharp fall in activity at alkaline pH values.

Adsorption to surfaces. The purified enzyme was only poorly adsorbed on to sand, sintered, powdered or scratched glass. In this respect the enzyme is like the penicillinase from *B. licheniformis* (Collins, 1964; Pollock, 1965*a*) but unlike those from *S. aureus* (Richmond, 1963) and *B. cereus* (Kogut *et al.* 1956).

Substrate-specificity and Michaelis constants. The purified enzyme was tested for hydrolysis of various penicillin and cephalosporin derivatives, and $V_{max.}$ and K_m values are shown in Table 2. The enzyme is somewhat similar to penicillinase from *B. licheniformis* strain 749 (Collins, 1964) but sharply different from the penicillinase of *S. aureus* (Richmond, 1963), since it has a higher affinity for methicillin than benzylpenicillin, although the $V_{max.}$ value is lower. In general the enzyme shows a broad specificity of action against both penicillin and cephalosporin derivatives. Thus cephaloridine is hydrolysed almost as rapidly as benzylpenicillin.

Inhibitors. A number of compounds that are known to inhibit enzymes were tested for their effect on this purified penicillinase. Unless otherwise stated the enzyme was incubated in the presence of 1mM-inhibitor for 15 min. at room temperature, and then assayed for enzyme activity. An equal quantity of enzyme, incubated without the inhibitor, acted as control. Disodium EDTA had no effect at pH 7·0 on the enzyme; *p*-chloromercuribenzoate produced about a 15% inhibition at 1mM. Smith (1963*a*) has reported that the penicillinase of *Aerobacter cloacae* is exceptional among penicillinases in being sensitive to *p*-chloromercuribenzoate. The *Aerobacter* enzyme is about 50% inhibited by 2μM-*p*-chloromercuribenzoate at pH 7·4, and this is about 500-fold less than the concentration needed to inhibit the enzyme from *E. coli* strain TEM by 15%. Probably, therefore, the enzyme in *E. coli* strain TEM contains no reactive thiol groups.

The enzyme from *E. coli* strain TEM is extremely

Table 2. *Kinetics of hydrolysis of various penicillin and cephalosporin derivatives by purified penicillinase from* E. coli

Experimental details are given in the Methods and Materials section. $V_{max.}$ values are expressed as moles of substrate hydrolysed/mole of enzyme/min. at 30° and pH 7·0. The $V_{max.}$ value for benzylpenicillin (2×10^4 moles of penicillin/mole of enzyme/min.) corresponds to a specific enzyme activity of 81 enzyme units/μg. of protein.

Substrate: approved name and chemical name	$V_{max.}$ (turnover no.)	K_m (μM)
Benzylpenicillin [6-(phenylacetamido)penicillanic acid]	2×10^4	22
Phenoxymethylpenicillin [6-(phenoxyacetamido)penicillanic acid]	1×10^4	—
Phenethicillin [DL-6-(α-phenoxypropionamido)penicillanic acid]	$0{\cdot}8 \times 10^4$	—
6-Aminopenicillanic acid	$1{\cdot}7 \times 10^4$	22
Methicillin [6-(2,6-dimethoxybenzamido)penicillanic acid]	2×10^2	3·2
Cloxacillin [6-(3-*o*-chlorophenyl-5-methylisoxazole-4-carboxamido)penicillanic acid]	$5{\cdot}4 \times 10^3$	
Ampicillin {6-[D(−)-α-aminophenylacetamido]penicillanic acid}	$2{\cdot}2 \times 10^4$	2·3
Quinacillin [6-(3-carboxyquinoxalin-2-ylamido)penicillanic acid]	6×10^2	260
Cephalosporin C [7-(5-amino-5-carboxyvaleramido)cephalosporanic acid]	$1{\cdot}6 \times 10^2$	—
Cephaloram (7-phenylacetamidocephalosporanic acid)	$3{\cdot}2 \times 10^3$	—
Cephaloridine {7-[(thien-2-yl)acetamido]-3-(pyrid-1-ylmethyl)-3-cephem-4-carboxylic acid betaine}	$1{\cdot}3 \times 10^4$	600

Table 3. *Comparison of the properties of purified penicillinase preparations from different species*

Data are taken from Pollock *et al.* (1956), Kogut *et al.* (1956) and M. R. Pollock (personal communication) for *B. cereus*, from Collins (1964) and Pollock (1965*a*) for *B. licheniformis* and from Richmond (1963, 1965) for *S. aureus*. 'Physiological efficiency' is defined by Pollock (1965*b*).

	Mol. wt.	Turnover no. (moles of substrate hydrolysed/ mole of enzyme/min.)	'Physiological efficiency' ($V_{max.}/K_m$)	Wt. of penicillinase/ dry wt. of organisms (%)
Gram-negative: *E. coli* (strain TEM)	16700	$1{\cdot}9 \times 10^4$	$3{\cdot}6 \times 10^6$	0·1
Gram-positive: *B. cereus* (569/H)	31600	$1{\cdot}6 \times 10^5$	$5{\cdot}9 \times 10^6$	1·1
B. cereus (5)	35700	$1{\cdot}5 \times 10^5$	$4{\cdot}9 \times 10^6$	0·9
B. licheniformis (749/C)	28000	$1{\cdot}1 \times 10^5$	$6{\cdot}6 \times 10^6$	1·2
B. licheniformis (6346/C)	28000	$2{\cdot}1 \times 10^4$	$5{\cdot}7 \times 10^6$	1·1
S. aureus (types A and C): constitutive mutant	29600	$2{\cdot}0 \times 10^4$	$1{\cdot}6 \times 10^7$	0·75–0·9

sensitive to iodine–potassium iodide solution. The enzyme was about 50% inhibited by a solution made 30μN with respect to iodine, and was completely inhibited within 2min. by a solution made 1mN with respect to iodine.

DISCUSSION

The main difference, so far, between the penicillinase made by *E. coli* strain TEM and the other penicillinases that have been purified is the size of the molecule. At a value of approx. 16700, the molecular weight of this penicillinase is about half that of the other penicillinases (Table 3). In terms of turnover numbers, however, the enzyme from *E. coli* is similar in specific activity against benzylpenicillin to the enzyme from *S. aureus* rather than those from the *Bacillus* spp. (Table 3), all but one of which are about 6–10 times as active. Pollock (1965*b*) has introduced the term 'physiological efficiency' (defined as $V_{max.}/K_m$) as a measure of the efficiency of an enzyme at hydrolysing various substrates under physiological conditions. By this parameter all the penicillinases are almost equally 'efficient' at hydrolysing benzylpenicillin under conditions where the substrate concentration is limiting (Table 3). A point where *E. coli* is clearly distinct from the Gram-positive species is in the amount of penicillinase protein synthesized by the culture when unrepressed. An enzyme content of

80 enzyme units/mg. dry wt. of organisms corresponds to a penicillinase protein content of 1μg. of protein/mg. dry wt. of bacteria, and this proportion of the total dry weight of the organisms (0·1%) is about one-tenth of the value found in the Gram-positive species (Table 3).

A number of crude preparations of penicillinases from Gram-negative species have been tested for their relative rates of hydrolysis of penicillin and cephalosporin derivatives (e.g. Smith, 1963*a*,*b*; Smith & Hamilton-Miller, 1963*a*,*b*; Hamilton-Miller, 1963; Fleming, Goldner & Glass, 1963), but it is not possible to identify for certain the enzyme studied here with any of those enzymes. This enzyme is clearly not the one studied in *Aerobacter cloacae* (now called *Enterobacter cloacae*) (Smith, 1963*a*) because of its insensitivity to *p*-chloromercuribenzoate, and it is also different from the one studied in *E. coli* strain 214T (Smith, 1963*b*) because of the clear differences between the relative rates of hydrolysis of benzylpenicillin and 6-aminopenicillanic acid by the two enzymes. Nor is it the enzyme found in *Enterobacter cloacae* (Fleming *et al.* 1963), which is predominantly a cephalosporinase and only slightly active against benzylpenicillin. The enzyme described in the present paper is most similar to those studied in *Klebsiella* (*Aerobacter*) *aerogenes* by Smith & Hamilton-Miller (1963*b*; Hamilton-Miller, 1963). The fact that a penicillinase in *E. coli* should be similar to an enzyme in *K. aerogenes* is not surprising, since the enzyme synthesis in *E. coli* strain TEM is mediated by an R-factor that is known readily to infect other genera of Enterobacteriaceae (Datta & Kontomichalou, 1965). Unfortunately, it is not yet known whether the penicillinase genes in the *K. aerogenes* strains studied by Smith & Hamilton-Miller (1963*a*,*b*) were carried on R-factors.

It is likely that penicillinase genes in Gram-negative species can either be chromosomal or carried on R-factors (Datta, 1965), and in view of the infectivity of R-factors among the Enterobacteriaceae it is important that no penicillinase should be regarded as 'species-specific' until its synthesis is proved to be controlled by chromosomal genes.

We acknowledge the excellent technical assistance of Mr Peter Thompson and Mr Keith Frayn. We are indebted to Beecham Research Laboratories for a gift of methicillin, ampicillin, cloxacillin and 6-aminopenicillanic acid, to Glaxo Laboratories for gifts of all the cephalosporin derivatives and to Boots Pure Drugs Ltd. for a gift of quinacillin.

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Formation of Glucose from Hexoses, Pentoses, Polyols and Related Substances in Kidney Cortex

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(*Received* 23 *June* 1965)

1. Slices of rat kidney cortex, on incubation in a saline medium, formed D-glucose from the following substances: D-fructose, D-galactose, D-mannose, L-sorbose, L-arabinose, D-xylose, glycerol, *myo*-inositol, L-iditol, sorbitol, xylitol, ribitol, methylglyoxal, dihydroxyacetone, L-glyceraldehyde, D-glyceraldehyde, DL-glyceraldehyde, DL-glycerate. Values for the rates of glucose formation from these precursors are given. 2. No glucose was formed from L-rhamnose, D-arabitol, D-arabinose, D-ribose, L-fucose, D-lyxose, mannitol, dulcitol, D-glucuronate, propane-1,2-diol and propan-2-ol. 3. The pathways of glucose formation from the various precursors are discussed (Scheme 1). 4. L-Glyceraldehyde inhibited the formation of glucose from D-glyceraldehyde.

Measurements of the rate of formation of glucose from non-carbohydrate precursors by slices of kidney cortex have been reported previously (Krebs, Bennett, de Gasquet, Gascoyne & Yoshida, 1963; Krebs & Yoshida, 1963). The present paper reports that rat kidney cortex forms glucose also from several hexoses, pentoses, trioses, polyols and related substances. The rates of formation of glucose from these precursors in kidney cortex are of the same order of magnitude as those in liver, an organ that converts various carbohydrates into glucose (Cori & Shine, 1935, 1936; Cori, Ochoa, Slein & Cori, 1951; Mendeloff & Weichselbaum, 1953).

EXPERIMENTAL

The general procedure of measuring the formation of glucose in kidney-cortex slices was as described by Krebs *et al.* (1963). Male Wistar rats weighing 200–300g. and fed on standard rat cubes were used. The washed slices were incubated for 1hr. at 40°. The substrate concentration was 10mM unless otherwise stated. D-Glucose was determined by the glucose-oxidase method, and all substrates were tested for contamination with glucose and whether they reacted with the glucose-oxidase reagents. The samples of mannose, xylose and galactose contained small amounts of glucose (1·5, 0·4 and 0·5% respectively) and corrections were made. Dihydroxyacetone gave a slight colour with the glucose-oxidase reagents for which an allowance was made in the calculation of the results.

RESULTS

Rate of formation of glucose from various precursors in rat kidney cortex. The rates of formation of glucose are shown in Table 1. Fructose formed glucose more rapidly than any of the other substrates tested. Next in effectiveness and approximately equally active were D-glyceraldehyde and

Table 1. *Glucogenesis from carbohydrates and related substrates in rat kidney-cortex slices*

For experimental details see Krebs *et al.* (1963) and the text. About 10mg. (dry wt.) of tissue was incubated in a Warburg vessel for 1hr. at 40° in 4ml. of the saline medium of Krebs & Henseleit (1932). The gas phase was O_2+CO_2 (95:5). The substrate concentration was 10mM. The results are given as means ±S.E.M. with the number of observations in parentheses.

Substrate added	Glucose formed (μmoles/g. dry wt./hr.)
None	15± 1·6 (8)
L-Lactate	103±12·0 (5)
D-Fructose	423±15·2 (4)
D-Galactose	56±14·8 (4)
D-Mannose	50± 6·4 (5)
L-Sorbose	79± 7·6 (4)
L-Arabinose	24± 2·7 (4)
D-Xylose	40± 4·5 (4)
Glycerol	79± 6·6 (5)
myo-Inositol	41± 2·9 (4)
L-Iditol	25± 3·9 (3)
Sorbitol	80± 7·6 (4)
Xylitol	106± 6·8 (9)
Ribitol	36± 3·5 (4)
Methylglyoxal	33± 3·1 (4)
Dihydroxyacetone	268±18·4 (4)
L-Glyceraldehyde	77± 7·3 (4)
DL-Glyceraldehyde	153± 5·6 (11)
D-Glyceraldehyde	322±19·9 (4)
DL-Glycerate	72± 9·8 (4)

dihydroxyacetone. Less reactive, but still giving a relatively high rate, was xylitol, which formed glucose about as rapidly as did lactate. Sorbitol, D-galactose, D-mannose, L-sorbose and DL-glycerate gave lower rates of formation of glucose. Still lower were the rates with L-iditol, *myo*-inositol, L-arabinose, ribitol and methylglyoxal. No significant increases (not recorded in Table 1) were obtained with L-rhamnose, D-arabitol, D-arabinose, D-ribose, L-fucose, D-lyxose, mannitol, dulcitol, D-glucuronate, propane-1,2-diol and propan-2-ol.

Inhibition by L-*glyceraldehyde.* L-Glyceraldehyde reacted much more slowly than D-glyceraldehyde. As the initial stages of the pathways for the D- and L-isomers are different (see Scheme 1), the addition of the DL-compound might be expected to show an additive effect. In fact DL-glyceraldehyde (whether prepared by mixing the D- and L-isomers or used as a commercial preparation) gave lower rates than D-glyceraldehyde (Table 1). These findings indicate that L-glyceraldehyde inhibits the formation of glucose from D-glyceraldehyde. This is further illustrated for various conditions by the results given in Table 2. L-Glyceraldehyde (20mM) did not inhibit appreciably the formation of glucose from lactate and fumarate. Since L-glyceraldehyde does not affect the pathway from lactate to glucose, the site of its inhibitory effect might be the stage causing the phosphorylation of D-glyceraldehyde. It is also possible that L-glyceraldehyde is phosphorylated (Bublitz & Kennedy, 1954) and that the L-glyceraldehyde 3-phosphate, or free L-glyceraldehyde, condenses with dihydroxyacetone phosphate to form sorbose 1,6-diphosphate or sorbose 1-phosphate respectively, which may exert inhibitory effects (see Lardy, Wiebelhaus & Mann, 1950; Tung, Ling, Byrne & Lardy, 1954).

Sheep kidney cortex. The precursors that form glucose in rat kidney cortex do so also in sheep kidney cortex (Table 3). The rates are in most cases about the same as in rat kidney. Exceptions

Table 2. *Inhibition of gluconeogenesis by* L-*glyceraldehyde in rat kidney-cortex slices*

Experimental conditions were as described in Table 1. Means ±S.E.M. are given with the number of experiments in parentheses; otherwise individual values are given.

Substrate(s) added (final concn.)	Glucose formed (μmoles/g. dry wt./hr.)
D-Glyceraldehyde (10mM)	228±18·8 (3)
L-Glyceraldehyde (10mM)	74± 9·7 (3)
D-Glyceraldehyde (10mM) +L-glyceraldehyde (10mM)	181±24 (3)
D-Glyceraldehyde (10mM)	280±12 (3)
L-Glyceraldehyde (20mM)	70±33·9 (3)
D-Glyceraldehyde (10mM) +L-glyceraldehyde (20mM)	82±34·3 (3)
D-Glyceraldehyde (10mM)	349
DL-Glyceraldehyde (20mM)	184
D-Glyceraldehyde (10mM) +DL-glyceraldehyde (20mM)	150
L-Lactate (10mM)	71
L-Glyceraldehyde (20mM)	96
L-Lactate (10mM)+L-glyceraldehyde (20mM)	159
Fumarate (10mM)	127
L-Glyceraldehyde (20mM)	49
Fumarate (10mM)+L-glyceraldehyde (20mM)	144

Table 3. *Formation of glucose from various precursors in sheep kidney cortex*

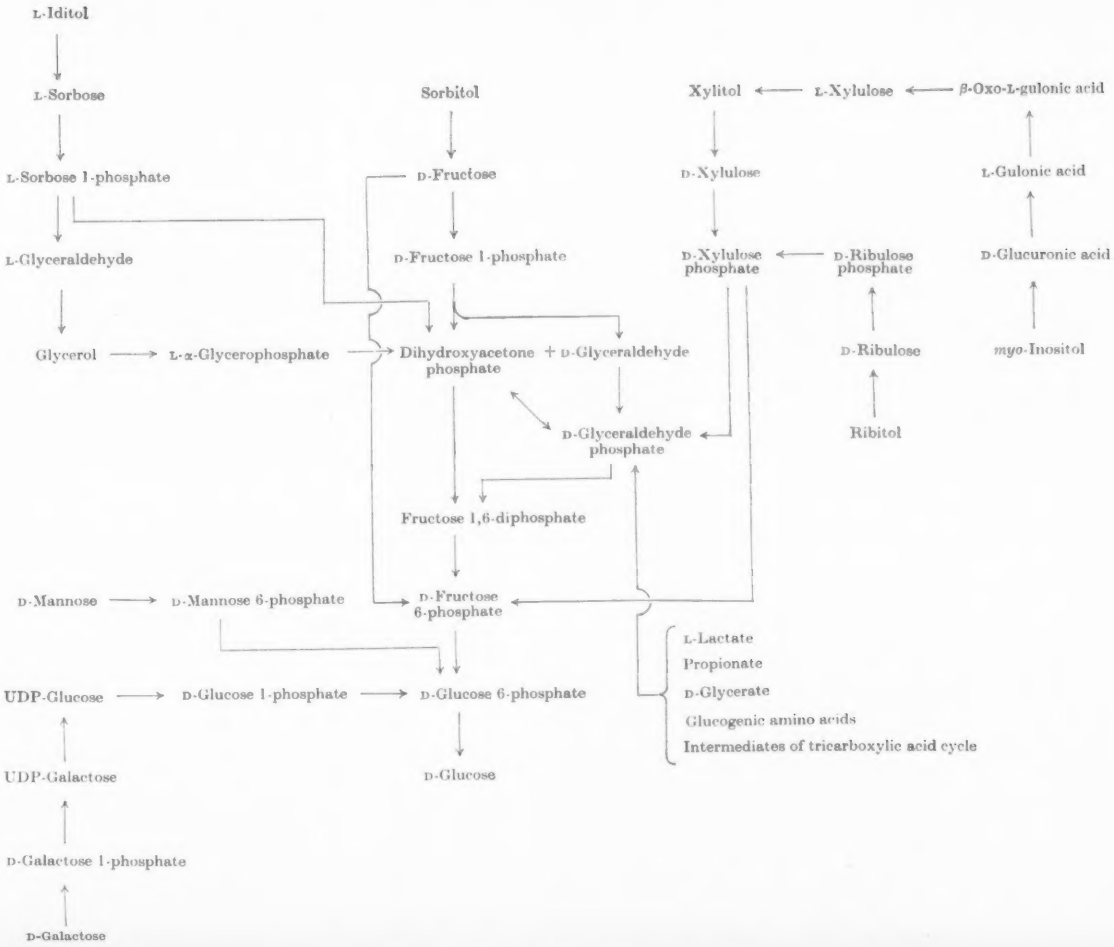
Conditions were as described in Table 1 for rat kidney-cortex slices. Freshly collected abattoir material was used. Values given are from single determinations.

Substrate added	Glucose formed (μmoles/g. dry wt./hr.) Expt. 1	Expt. 2
None	28	23
D-Fructose	152	192
D-Galactose	85	86
D-Mannose	53	—
L-Sorbose	—	41
L-Arabinose	43	—
D-Xylose	41	—
Glycerol	70	—
myo-Inositol	—	47
Sorbitol	52	59
Xylitol	95	99
Dihydroxyacetone	167	149
D-Glyceraldehyde	—	100

Table 4. *Effect of substrate concentration on the rate of glucose formation from polyols in rat kidney-cortex slices*

Experimental conditions were as described in Table 1 except for the substrate concentration. Means ±S.E.M. are given with the numbers of experiments in parentheses; otherwise average duplicate determinations are given.

Substrate added (final concn.)	Glucose formed (μmoles/g. dry wt./hr.)
Sorbitol (5mM)	47± 4·6 (3)
Sorbitol (10mM)	78± 2·8 (4)
Sorbitol (20mM)	125±12·1 (4)
Sorbitol (40mM)	134± 8·7 (3)
Xylitol (10mM)	120± 3·5 (3)
Xylitol (20mM)	160± 9·8 (3)
Xylitol (40mM)	169± 4·0 (3)
L-Iditol (10mM)	29
L-Iditol (20mM)	49
L-Iditol (40mM)	68



Scheme 1. Pathways of glucose formation from hexoses, pentoses, polyols and related substances. The intermediary stages leading from lactate, propionate, glucogenic amino acids and intermediates of the tricarboxylic acid cycle to glyceraldehyde phosphate are not shown (see Krebs, 1964). There are five steps

Scheme 1. Pathways of glucose formation from hexoses, pentoses, polyols and related substances. The intermediary stages leading from lactate, propionate, glucogenic amino acids and intermediates of the tricarboxylic acid cycle to glyceraldehyde phosphate are not shown (see Krebs, 1964). There are five steps leading from D-xylulose phosphate to fructose 6-phosphate and glyceraldehyde phosphate. They are catalysed by enzymes of the oxidative pentose phosphate cycle (xylulose phosphate epimerase, pentose phosphate isomerase, transketolase and transaldolase). The sum of these reactions is:

3 Xylulose phosphate → 2 fructose 6-phosphate + D-glyceraldehyde phosphate

References to the reactions shown in the scheme are the following: Embden & Griesbach (1914), Blakley (1951), Hickman & Ashwell (1956), Touster, Reynolds & Hutcheson (1956), Hollmann & Touster (1957), McCormick & Touster (1961), Touster & Shaw (1962) and Exton & Edson (1964) on hexitols and pentitols; Cadenas & Sols (1960), Kattermann *et al.* (1961) and DiPietro (1964) on fructose; Hers (1962), Kattermann *et al.* (1961) and Landau & Merlevede (1963) on glycerol, trioses and glycerate; Anderson & Coots (1958), Burns *et al.* (1959) and Charalampous (1960) on *myo*-inositol; Kalckar (1958) and Abraham, Fitch & Chaikoff (1961) on mannose and galactose. Most of the evidence is derived from experiments on liver.

are fructose, dihydroxyacetone and D-glyceraldehyde, which react more rapidly in rat kidney.

Effect of substrate concentration. In the previous experiments where lactate, amino acids, glycerol and intermediary metabolites were precursors of glucose (Krebs *et al.* 1963) optimum rates were obtained with 10mM-substrate. The rate of formation of glucose from polyols, in contrast, increases markedly when their concentration rises above 10mM (Table 4). Since the K_m of polyol dehydrogenase for the substrate is about 1mM (Smith, 1962), the effect of the high substrate concentration cannot be accounted for by a low affinity of the enzyme for the substrate. A rate-limiting factor for the formation of glucose may be the entry of the substrate into the cells, or alternatively the effect of the substrate concentrations could be explained by the presence in the tissue of another polyol dehydrogenase with a higher K_m. A multiplicity of NAD-dependent polyol dehydrogenases has been suggested by observations of Hollmann & Touster (1957).

DISCUSSION

It is already well established (see Cori & Shine, 1935, 1936; Exton & Edson, 1964; Kattermann, Dold & Holzer, 1961) that various polyols (sorbitol, xylitol), hexoses (galactose, mannose, fructose), pentoses (xylose, ribose, xylulose), trioses (dihydroxyacetone, glyceraldehyde) and *myo*-inositol can form glucose in the mammalian body, and that liver contains the required enzymes. The pathways leading from the various precursors to glucose are also known and are summarized in Scheme 1. The experiments reported in this paper show that kidney cortex is a highly active site of the formation of glucose from all substrates that are glucogenic in liver. Several of the necessary enzymes have been shown previously in kidney cortex. These include polyol dehydrogenase (L-iditol–NAD oxidoreductase, EC 1.1.1.14) (Blakley, 1951), the enzymes for the conversion of inositol into fructose (Burns, Trousof, Evans, Papadopoulos & Agranoff, 1959) and some of the enzymes initiating the metabolism of C_3 precursors, namely glycerol kinase or L-triokinase (Kalckar, 1938; Wieland & Suyter, 1957) and D-glycerate kinase (Holzer & Holldorf, 1957). The rapid formation of glucose from xylitol indicates that the transaldolase, transketolase and xylulosekinase reactions, which are intermediary stages in the formation of glucose from xylitol, can be relatively rapid in kidney cortex.

Polyols that are not attacked by polyol dehydrogenase in the liver (D-arabitol, mannitol, dulcitol; Blakley, 1951; McCorkindale & Edson, 1954) did not form glucose in kidney cortex. The failure of propane-1,2-diol to form glucose is relevant to the

hypothesis of a 'propanediol pathway' (Shull & Miller, 1960) of conversion of ketone bodies into carbohydrate.

The observed reaction rates are in general agreement with the pathways of Scheme 1 in that the postulated intermediates, whenever tested, react at least as rapidly as the starting materials. This applies to the pathways initiated by L-iditol, sorbitol and xylitol.

This work was supported by U.S. Public Health Service Grant no. AM08715. The authors are indebted to Dr P. W. Kent for a sample of L-iditol prepared by him, and to Mrs Janet Wright for technical assistance.

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Biochem. J. (1966) **98**, 215

The Metabolism of 3-Methylcholanthrene and some Related Compounds by Rat-Liver Homogenates

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(*Received* 22 *July* 1965)

1. A chromatographic investigation of the products of the metabolism of 3-methylcholanthrene by rat-liver homogenates showed the formation of compounds with the properties of 1- and 2-hydroxy-3-methylcholanthrene, *cis*- and *trans*-1,2-dihydroxy-3-methylcholanthrene and 11,12-dihydro-11,12-dihydroxy-3-methylcholanthrene. A glutathione conjugate that is probably *S*-(11,12-dihydro-12-hydroxy-3-methyl-11-cholanthrenyl)glutathione was also detected. 3-Methylcholanthrene-1- and -2-one and -1,2-quinone were also present, but these products may have arisen by the chemical oxidation of the corresponding hydroxy compounds. 2. Other metabolic products were tentatively identified as 9- and 10-hydroxy-3-methylcholanthrene, 4,5-dihydro-4,5-dihydroxy-3-methylcholanthrene and 3-hydroxymethylcholanthrene. 3. 1- and 2-Hydroxy-3-methylcholanthrene were converted by homogenates into the related ketones and into products with the properties of *cis*- and *trans*-1,2-dihydroxy-3-methylcholanthrene: 3-methylcholanthren-1- and -2-one were converted into their related hydroxy compounds and into the isomeric 1,2-dihydroxy compounds. The isomeric 1,2-dihydroxy compounds were each partly converted into the other isomer by these homogenates. All the above substrates also yielded products that appeared to be derivatives of 3-hydroxymethylcholanthrene. 4. 3-Methylcholanthrylene was converted by rat-liver homogenates into products with the properties of *trans*-1,2-dihydroxy-3-methylcholanthrene, 2-hydroxy-3-methylcholanthrene and 3-methylcholanthren-2-one. A small amount of the *cis*-1,2-dihydroxy compound was also formed, together with a glutathione conjugate that is possibly *S*-(2-hydroxy-3-methyl-1-cholanthrenyl)glutathione or its positional isomer. 5. An unidentified product was detected in the metabolism of 3-methylcholanthrene, the monohydroxy compounds, the ketones and the dihydroxy compounds, the formation of which appeared to involve metabolism at the 1,2-bond. 6. 11,12-Epoxy-11,12-dihydro-3-methylcholanthrene was converted by rat-liver homogenates into products with the properties of 11-hydroxy-3-methylcholanthrene (or, less likely, the 12-isomer), 11,12-dihydro-11,12-dihydroxy-3-methylcholanthrene and the glutathione conjugate described above. Products with the properties of these compounds were formed when the epoxide was allowed to react with glutathione in an aqueous medium. 7. Mouse-liver homogenate converted 3-methylcholanthrene into products with the chromatographic properties of 1- and 2-hydroxy-3-methylcholanthrene, *cis*- and *trans*-1,2-dihydroxy-3-methylcholanthrene, 11,12-dihydro-11,12-dihydroxy-3-methylcholanthrene, 3-methylcholanthrene-1- and -2-one and -1,2-quinone and the unidentified hydroxy-3-methylcholanthrenes. 8. The syntheses of *cis*- and *trans*-1,2-dihydroxy-3-methylcholanthrene, 3-methylcholanthren-2-one, 2-hydroxy-3-methylcholanthrene, 3-methylcholanthrylene, 11,12-epoxy-11,12-dihydro-3-methylcholanthrene and *trans*-11,12-dihydro-11,12-dihydroxy-3-methylcholanthrene are described.

Although 3-methylcholanthrene (III) has strong cancer-producing properties and has been the subject of many biological experiments, little is known of its fate in the animal body. Harper (1959) examined the faeces of mice that had been injected with the hydrocarbon and detected two metabolites, a phenol that was either 8- or 10-hydroxy-3-methylcholanthrene and a carboxylic acid that was

tentatively identified as cholanthrene-3-carboxylic acid. Evidence was also obtained for the presence of phenolic sulphuric acid and glucuronic acid conjugates. This paper describes the metabolism of 3-methylcholanthrene (III) by rat-liver homogenates.

EXPERIMENTAL

Melting points are uncorrected.

Spectra. Infrared spectra were measured as mulls in Nujol on a Perkin–Elmer Infracord. Ultraviolet-absorption spectra were measured in ethanol, except where indicated below, on a Unicam SP.500 spectrophotometer or on a Perkin–Elmer model 137 spectrophotometer. The spectra of compounds eluted from paper or from thin-layer chromatograms were usually measured on the latter instrument, solutions being obtained by eluting with methanol containing 1% (v/v) of aq. NH_3 (sp.gr. 0·88) the appropriate bands cut from paper chromatograms, or by eluting with ethanol the appropriate bands removed from thin-layer chromatograms. The positions of the bands were usually determined by the fluorescence seen when the chromatograms were examined by u.v. light.

Chromatography. Paper chromatography was carried out on Whatman no. 1 chromatography paper by downward development for 18hr. either in (1) butan-1-ol–propan-1-ol–aq. 2N-NH_3 (2:1:1, by vol.) or in (2) butan-1-ol–acetic acid–water (2:1:1, by vol.). The dried chromatograms were examined in u.v. light and either dipped in a solution of 0·2% ninhydrin in acetone or in the platinic iodide reagent of Toennies & Kolb (1951).

Thin-layer chromatograms were prepared by coating glass plates (20cm.×20cm.) with layers of silica gel G (E. Merck A.-G., Darmstadt, Germany) of 0·25mm. thickness. The chromatograms were developed for 10cm. with (a) light petroleum (b.p. 60–80°)–benzene (19:1, v/v), (b) benzene or (c) benzene–ethanol (19:1, v/v). They were examined in u.v. light while still wet, both before and after exposure to NH_3, and were then sprayed with conc. HCl and heated at 80° for 10min. The two-dimensional chromatograms referred to below were developed in the first direction for 10cm. with solvent (c), sprayed with conc. HCl and heated at 100° for 10min. and developed in the second direction for 10cm. with solvent (b). In this way, products arising from the decomposition of acid-labile precursors were detected. The properties on thin-layer chromatograms described below are listed in Table 1.

Table 1. *Properties of compounds related to 3-methylcholanthrene on thin-layer chromatograms*

Details are given in the text.

Compound	R_F in Benzene	R_F in Benzene–ethanol (19:1, v/v)	Fluorescence	Colour after spraying with conc. HCl and heating at 100°
1-Hydroxy-3-methylcholanthrene (X)	0·20	0·62	Violet	Pale brown
1-Acetoxy-3-methylcholanthrene	0·49	0·90	Violet	Pale brown
2-Hydroxy-3-methylcholanthrene (VIII)	0·15	0·55	Violet	Pale brown
2-Acetoxy-3-methylcholanthrene	0·49	0·90	Violet	Pale brown
3-Methylcholanthren-1-one (XIII)	0·46	0·90	Green, turning orange	Pale yellow*
3-Methylcholanthren-2-one (XII)	0·46	0·90	Yellow	Pale yellow*
3-Methylcholanthrene-1,2-quinone (IV)	0·18	0·82	Olive green	Yellow*
cis-1,2-Dihydroxy-3-methylcholanthrene (VII)	0·00	0·28	Violet	Yellow†
cis-1,2-Diacetoxy-3-methylcholanthrene	0·13	0·70	Violet	Yellow†
trans-1,2-Dihydroxy-3-methylcholanthrene (VII)	0·00	0·23	Violet	Yellow†
trans-1,2-Diacetoxy-3-methylcholanthrene	0·18	0·77	Violet	Yellow†
cis- and *trans*-11,12-Dihydro-11,12-dihydroxy-3-methylcholanthrene (V)	0·00	0·23	Dark violet	Brown
Phenol, probably 11-hydroxy-3-methylcholanthrene (I, R=H)	0·49	0·92	Violet (yellow‡)	Brown
11,12-Epoxy-11,12-dihydro-3-methylcholanthrene (II)	0·39	0·92	Dark violet, turning violet (yellow‡)	Brown
3-Methylcholanthrene (III)§	0·95	0·95	Violet	Pale yellow
3-Methylcholanthrylene (XIV)§	0·95	0·95	Dark absorption	Yellow*
2-Acetoxy-3-methylcholanthrylene	0·55	0·90	Dark absorption	Orange, then brown*
Methyl 9-methylbenz[*a*]anthracene-7,12-quinone-8-acetate (VI, R=Me)	0·31	0·85	Orange–yellow	Yellow*
Methyl 9-methylbenz[*a*]anthracene-7,12-quinone-8-carboxylate (XI, R=Me)	0·35	0·90	Orange	Yellow*

* These colours were seen on untreated chromatograms.

† This colour is due to the formation of 3-methylcholanthren-2-one (XII).

‡ Fluorescence in the presence of NH_3.

§ R_F 0·45 in light petroleum (b.p. 60–80°)–benzene (19:1, v/v).

The small-scale chromic acid oxidations described below were carried out as follows. About 1mg. of solid, or the material obtained by eluting with ether bands removed from thin-layer chromatograms, was heated on the steam bath with 5% (w/v) of $Na_2Cr_2O_7$ in acetic acid (0·1–0·5ml.) for 15min. The mixture was diluted with water and kept at room temperature until solid material separated. This was collected by centrifugation, washed with a little water and suspended in methanol (1ml.). An excess of diazomethane in ether was added and, at the end of the reaction, the solvents were evaporated. The residue was examined on thin-layer chromatograms developed with solvent (b) or (c).

Materials

3-*Methylcholanthrene* (III). This was obtained from Eastman Kodak Ltd. (Kirkby, Liverpool) and from Roche Products Ltd. (Welwyn Garden City, Herts.). Its light-absorption curve showed $\lambda_{max.}$ at 222, 236, 260, 274, 283, 294, 326, 342, 357, 374 and 391 mμ.

Methyl 9-methylbenz[*a*]*anthracene-7,12-quinone-8-acetate* (VI, R=Me) *and 8-carboxylate* (XI, R=Me). The parent acid was prepared by heating the hydrocarbon (III) (1g.) under reflux with $Na_2Cr_2O_7$ (5g.) in acetic acid (25ml.) (Cook & Haslewood, 1934). The acid, suspended in methanol, was esterified with diazomethane in ether and the product recrystallized from methanol to give methyl 9-methylbenz[*a*]anthracene-7,12-quinone-8-acetate (VI, R=Me) (280mg.) as yellow needles, m.p. 217° (Found: C, 76·5; H, 4·65. Calc. for $C_{22}H_{16}O_4$: C, 76·7; H, 4·7%); $\lambda_{max.}$ at 254, 285 and 346 mμ (log ϵ 4·30, 4·70 and 3·70 respectively). Bachmann (1937) reports m.p. 213–214° and Fieser & Hershberg (1938), m.p. 221·5–222° (corr.). The mother-liquors were evaporated and the dry residue was chromatographed in benzene on a silica gel column (20g.) (200–300 mesh; Koch–Light Laboratories Ltd., Colnbrook, Bucks.). The two yellow bands were eluted separately and the eluates evaporated. The residue from the faster-moving band, recrystallized from methanol, gave a product (15mg.) that is probably *methyl 9-methylbenz*[a]*anthracene-7,12-quinone-8-carboxylate* (XI, R=Me) in yellow needles, m.p. 178° (Found: C, 76·3; H, 4·2. $C_{21}H_{14}O_4$ requires C, 76·35; H, 4·3%); $\lambda_{max.}$ at 251, 288 and 338 mμ (log ϵ 4·28, 4·59 and 3·59 respectively). The residue from the slower-moving band, recrystallized from methanol, yielded methyl 9-methylbenz[*a*]anthracene-7,12-quinone-8-acetate (VI, R=Me) (30mg.) as yellow needles, m.p. and mixed m.p. 217°.

3-*Methylcholanthren-1-one* (XIII). This was prepared from (III) and $Na_2Cr_2O_7$ in cold acetic acid by the method of Fieser & Hershberg (1938). The crude ketone was purified by chromatography in benzene on a Florisil (100–200 mesh; Koch–Light Laboratories Ltd.) column. The pale-yellow band was eluted, the solvent evaporated and the residue recrystallized from acetic acid to yield the ketone (XIII) in yellow needles, m.p. 258° [Fieser & Hershberg (1938) report m.p. 262–263° (corr.) and Badger *et al.* (1940) m.p. 256–257°]; $\lambda_{max.}$ at 233·5, 257, 269, 296, 307·5, 367, 387 and 403 mμ (log ϵ 4·64, 4·39, 4·35, 4·66, 4·68, 3·84, 4·02 and 3·99 respectively). The infrared spectrum showed strong carbonyl absorption at 1700cm.$^{-1}$. A little of the ketone (XIII) was oxidized with $Na_2Cr_2O_7$ in acetic acid and the product methylated as described above. Products with the mobilities and properties on thin-layer chromatograms developed with solvents (b) and (c) of the esters (VI, R=Me) and (XI, R=Me) were detected, the former being the major product.

1-*Hydroxy-3-methylcholanthrene* (X). The ketone (XIII) (100mg.) and lithium tetrahydroaluminate (500mg.), in dry ether (250ml.), were heated under reflux for 6hr. Water (50ml.), followed by 2N-H_2SO_4 (20ml.), was added and the mixture shaken. The ether layer was separated, washed with water, dried over Na_2SO_4 and the ether evaporated. The residue was recrystallized from benzene to yield 1-hydroxy-3-methylcholanthrene (X) (80mg.) in needles, m.p. 211–212° (decomp.) (Found: C, 88·4; H, 6·0. Calc. for $C_{21}H_{16}O$: C, 88·7; H, 5·7%); $\lambda_{max.}$ at 236, 263·5, 273·5, 284, 295, 325, 340, 356, 374 and 391 mμ (log ϵ 4·50, 4·54, 4·78, 4·86, 4·95, 3·64, 3·83, 3·90, 3·73 and 3·01 respectively) and an inflexion at 303 mμ. The melting points (corr.) of the products obtained by Fieser & Hershberg (1938) from the oxidation of the hydrocarbon (III) with lead tetra-acetate and hydrolysis of the acetoxy derivative varied between 207–209° (decomp.) and 221–222° (decomp.). Attempts to obtain the hydroxy derivative (X) by this route were unsuccessful, for, although the corresponding acetoxy derivative was detected on thin-layer chromatograms, chromatography of the reaction mixtures on silica-gel- or on Florisil-packed columns led to extensive decomposition with the formation of coloured products including 3-methylcholanthrylene (XIV) described below.

When a portion of the hydroxy derivative (X) was oxidized with $Na_2Cr_2O_7$ and the product methylated as before, the methyl ester (VI, R=Me) and a small amount of the ester (XI, R=Me) were detected on thin-layer chromatograms.

The acetate, prepared from the hydroxy derivative (X) with acetic anhydride in pyridine, separated from ethanol in needles, m.p. 178° [Fieser & Hershberg (1938) report m.p. 177·5–178·5° (corr.)], $\lambda_{max.}$ at 224, 235, 262, 273, 283, 294, 325, 339, 355, 375 and 391 mμ and an inflexion at 303 mμ.

3-*Methylcholanthrylene* (XIV). 3-Methylcholanthrene (III) (5g.), in dry carbon tetrachloride (1l.), was cooled to 0° and bromine (1·1ml.) in carbon tetrachloride (50ml.) was added during 15min. Pyridine (50ml.) was added and, after 1hr., the mixture was filtered to remove pyridine hydrobromide. The solvent was removed under reduced pressure and the residue extracted twice with boiling light petroleum (b.p. 80–100°) (150ml.) to leave a brown residue that was not examined. The extract was cooled and the product that separated recrystallized from light petroleum (b.p. 80–100°) to yield 3-*methylcholanthrylene* (XIV) (1·7g.) in orange plates with a metallic lustre, m.p. 194° (Found: C, 94·2; H, 5·3. $C_{21}H_{14}$ requires C, 94·7; H, 5·3%); $\lambda_{max.}$ at 262, 292, 314, 384 and 398 mμ (log ϵ 4·61, 4·40, 4·43, 4·04 and 3·99 respectively) and inflexions at 281, 305 and 366 mμ. The hydrocarbon (XIV) (10mg.) was oxidized at 100° for 15min. with $Na_2Cr_2O_7$ (50mg.) in acetic acid (1ml.), and the product esterified with diazomethane as before, to yield methyl 9-methylbenz[*a*]anthracene-7,12-quinone-8-carboxylate (XI, R=Me) in yellow needles from methanol, m.p. and mixed m.p. 175–176°.

Oxidation of 3-*methylcholanthrene* (III) *with lead tetra-acetate.* 3-Methylcholanthrene (III) (3g.), in acetic acid (350ml.), was heated at 100° with lead tetra-acetate (10g.) for 30min. The solution was poured into water (850ml.) and the solid collected and dried and chromatographed in

benzene on a Florisil (200g., 100–200 mesh) column to yield four main fractions. The fastest-moving of these formed an orange band: the eluates containing the material forming this fraction were evaporated and the residue was recrystallized three times from light petroleum (b.p. 80–100°) to yield 3-methylcholanthrylene (XIV) (85mg.) in orange plates with a metallic lustre, m.p. and mixed m.p. 194°.

The second fraction formed a pale-orange band on the column. The band was eluted and the solvent evaporated to yield a red solid that was dissolved in the minimum amount of boiling benzene. The solution was kept overnight at 0° and the red solid (15mg.) that separated was recrystallized from benzene to yield a product that is probably *2-acetoxy-3-methylcholanthrylene* in red plates with a metallic lustre that softened and decomposed at 180–184° (Found: C, 84·7; H, 5·0. $C_{23}H_{16}O_2$ requires C, 85·2; H, 5·0%); $\lambda_{max.}$ at 218, 264, 290, 314, 388 and 410mμ (log ε 4·57, 4·62, 4·46, 4·44, 4·02 and 3·99 respectively) and inflexions at 305 and 365mμ. The infrared spectrum showed carbonyl absorption at 1770cm.$^{-1}$. When a little of the acetate was oxidized with $Na_2Cr_2O_7$ and the product methylated as before, a compound indistinguishable from the ester (XI, R=Me) was detected on thin-layer chromatograms. A little of the acetate was heated to boiling for 2min. with 5% (w/v) KOH in methanol (0·5ml.). A bright magenta solution was obtained, the colour of which was discharged when it was diluted with water and acidified with conc. HCl. An ether extract of this solution was found to contain a number of products, one of which was indistinguishable in its mobility on thin-layer chromatograms developed with solvent (b) and in its fluorescence in u.v. light from 3-methylcholanthren-2-one (XII) described below.

The third fraction, which formed a pale-yellow band, yielded 3-methylcholanthren-1-one (XIII) on evaporation of the eluting solvent, separating from acetic acid in yellow needles (250mg.), m.p. and mixed m.p. 258°.

The fourth fraction was eluted from the column with benzene–ethanol (99:1, v/v) as a dark-coloured band. Evaporation of the solvent yielded a brown gum that appeared to be a mixture of acetoxy derivatives, together with unidentified oxidation products. The gum was heated under reflux with KOH (2g.) in methanol (200ml.) for 15min. and the mixture poured into water (750ml.) and the solution extracted three times with ether (250ml.) to leave a dark-green fluorescent aqueous layer. The residue obtained on evaporation of the ether was chromatographed in benzene–ethanol (99:1, v/v) on a Florisil column (100g.). After the elution of a dark-coloured band that contained unidentified products, two bands, which were detected by their violet fluorescence in u.v. light, were successively eluted, followed by one that was detected by its yellow colour in daylight.

The solution containing the material forming the faster-moving of the fluorescent bands was evaporated: the dark solid was recrystallized three times from aq. ethanol to yield a product (150mg.) that, for reasons discussed below, appeared to be cis-*1,2-dihydroxy-3-methylcholanthrene* (VII) in pale-green needles, m.p. 225° (Found: C, 83·7; H, 5·5. $C_{21}H_{16}O_2$ requires C, 84·0; H, 5·4%); $\lambda_{max.}$ at 223·5, 232, 262, 273, 284, 294·5, 324, 338, 355 and 374mμ (log ε 4·72, 4·65, 4·59, 4·64, 4·88, 4·97, 3·67, 3·84, 3·91 and 3·76 respectively). Acetylation with acetic anhydride in pyridine yielded cis-*1,2-diacetoxy-3-methylcholanthrene*, separating from ethanol in needles, m.p. 217° (Found: C, 77·8; H, 5·2. $C_{25}H_{20}O_4$ requires C, 78·1; H, 5·2%); $\lambda_{max.}$ at 222·5, 234, 261, 273, 283, 294, 324, 338, 354 and 372mμ (log ε 4·35, 4·46, 4·47, 4·62, 4·90, 5·01, 3·72, 3·90, 3·97 and 3·82 respectively).

The solution containing the material forming the second fluorescent band was evaporated to a yellow solid that was recrystallized three times from aq. ethanol to yield trans-*1,2-dihydroxy-3-methylcholanthrene* (VII) (250mg.), in pale-green needles, m.p. 206° (Found: C, 83·5; H, 5·2%); $\lambda_{max.}$ at 223, 232, 262, 272·5, 283, 294, 324, 338, 354·5 and 374mμ (log ε 4·72, 4·66, 4·58, 4·66, 4·90, 4·99, 3·66, 3·82, 3·89 and 3·72 respectively). trans-*1,2-Diacetoxy-3-methylcholanthrene* separated from ethanol in prismatic needles, m.p. 202° (Found: C, 77·9; H, 5·3%); $\lambda_{max.}$ at 222·5, 234, 261, 273, 283, 294, 324, 338, 354 and 372mμ (log ε 4·66, 4·49, 4·48, 4·62, 4·89, 5·00, 3·72, 3·90, 3·98 and 3·86 respectively).

When small amounts of the dihydroxy compounds were oxidized and the products methylated as described above, only the ester (XI, R=Me) was detected on thin-layer chromatograms developed with solvent (b) or (c). When tested with the potassium triacetylosmate reagent of Criegee, Marchand & Wannowius (1942), the dihydroxy compound of m.p. 225° rapidly changed the colour of the reagent from blue to purple and then to grey, whereas the dihydroxy compound of m.p. 206° had no effect. This indicates that the former dihydroxy compound probably has the *cis*- and the latter the *trans*-configuration. Supporting evidence for this is discussed below.

Evaporation of the solution containing the material forming the yellow band yielded a solid that was recrystallized from acetic acid to give a product that was probably *3-methylcholanthrene-1,2-quinone* (IV) (45mg.) in pale-orange needles, m.p. 346° (Found: C, 85·1; H, 4·3. $C_{12}H_{12}O_2$ requires C, 85·1; H, 4·1%); $\lambda_{max.}$ at 221, 234, 251, 289, 296, 390 and 410mμ. The infrared spectrum showed strong carbonyl absorption at 1705cm.$^{-1}$. The quinone (IV) was recovered unchanged (mixed m.p.) after an attempted oxidation with $Na_2Cr_2O_7$ in acetic acid.

3-Methylcholanthren-2-one (XII). When either the *cis*- or the *trans*-isomer of the dihydroxy compound (VII) (100mg.), in acetic acid (1ml.) containing either a few drops of conc. HCl or of water, was heated at 100° for 5min. the solution became yellow. Water was added until the solution was cloudy and it was then allowed to cool. In each case, the crystals (about 70mg.) were recrystallized from acetic acid to yield *3-methylcholanthrene-2-one* (XII) in yellow needles, m.p. 205° (decomp.) (Found: C, 89·0; H, 4·75. $C_{21}H_{14}O$ requires C, 89·3; H, 5·0%); $\lambda_{max.}$ at 245·5, 277, 295, 302, 309·5, 367, 385 and 400mμ (log ε 4·46, 4·59, 4·63, 4·61, 4·67, 3·69, 3·79 and 3·73 respectively). The infrared spectrum of (XII) showed strong carbonyl absorption at 1700cm.$^{-1}$.

3-Methylcholanthren-2-one (XII) (50mg.) and $Na_2Cr_2O_7$ (250mg.), in acetic acid (5ml.), were heated under reflux for 30min. The solution was poured into water and the product suspended in methanol and treated with an excess of diazomethane in ether. The solvents were removed and the residue was recrystallized three times from methanol to yield methyl 9-methylbenz[*a*]anthracene-7,12-quinone-8-carboxylate (XI, R=Me) (25mg.) in yellow needles, m.p. and mixed m.p. 175–176°. The residue obtained on evapora-

tion of the mother-liquors was examined on thin-layer chromatograms developed with solvent (b), when a product, R_F 0·48, with a violet fluorescence in u.v. light, was detected whose u.v. spectrum showed $\lambda_{max.}$ 223, 233, 261, 271, 282, 292, 336, 352, 367 and 394 mμ. The compound was not investigated further, but it could be the dimethyl ester of 9-methylbenz[*a*]anthracene-7,8-dicarboxylic acid, which might be expected from the oxidation of the 1,2-bond of the ketone (XII).

2-Hydroxy-3-methylcholanthrene (VIII). 3-Methylcholanthren-2-one (XII) (100 mg.) was reduced with lithium tetrahydroaluminate (500 mg.) in ether as described above. The product was recrystallized from benzene to yield *2-hydroxy-3-methylcholanthrene* (VIII) (80 mg.) in needles, m.p. 186° (decomp.) (Found: C, 89·0; H, 5·8. $C_{21}H_{16}O$ requires C, 88·7; H, 5·7%); $\lambda_{max.}$ at 223, 237, 262, 276, 284, 295, 304, 324·5, 340, 356·5, 375 and 391 mμ (log ε 4·49, 4·45, 4·48, 4·54, 4·81, 4·91, 4·18, 3·61, 3·79, 3·85, 3·78 and 3·04 respectively). The *acetate*, prepared from the hydroxy compound (VIII) with acetic anhydride in pyridine, separated from ethanol in needles, m.p. 174° (Found: C, 84·7; H, 5·7. $C_{23}H_{18}O_2$ requires C, 84·6; H, 5·6%); $\lambda_{max.}$ at 221, 235, 260, 273, 284, 295, 303, 325, 340, 356, 375 and 391 mμ (log ε 4·89, 4·47, 4·70, 4·70, 4·92, 5·02, 4·15, 3·66, 3·85, 3·91, 3·74 and 3·03 respectively). When a little of the hydroxy compound (VIII) was oxidized with $Na_2Cr_2O_7$ and the product methylated, a compound with the properties of the ester (XI, R = Me) was detected on thin-layer chromatograms. The ester (VI, R = Me) was not detected.

Alternative methods of preparation of the isomeric dihydroxy compounds (VII). 1-Acetoxy-3-methylcholanthrene (100 mg.) and lead tetra-acetate (140 mg.), in acetic acid (5 ml.), were heated at 100° for 30 min. The solution was poured into water and the solid heated under reflux with KOH (100 mg.) in methanol (5 ml.) for 10 min. The solution was poured into water (100 ml.) and the mixture extracted three times with ether (25 ml.). The ether was evaporated and the residue chromatographed on six thin-layer chromatograms developed in solvent (c). Bands at R_F 0·28 and 0·20, detected by their violet fluorescence in u.v. light, were removed and the absorbed material was eluted with ether. The ether was evaporated and the residues were recrystallized from aqueous ethanol. That derived from the faster-moving bands yielded *cis*-1,2-dihydroxy-3-methylcholanthrene (12 mg.) as pale-green needles, m.p. and mixed m.p. 223–225°, and that from the slower-moving band yielded *trans*-1,2-dihydroxy-3-methylcholanthrene (25 mg.) as pale-green needles, m.p. and mixed m.p. 205°.

2-Acetoxy-3-methylcholanthrene (10 mg.) and lead tetra-acetate (15 mg.), in acetic acid (1 ml.), were similarly allowed to react. The product was hydrolysed with KOH in methanol and the hydrolysate examined on thin-layer chromatograms developed with solvent (c). Products with the mobilities and properties of *cis*- and *trans*-1,2-dihydroxy-3-methylcholanthrene (VII) were detected, the latter isomer forming the larger spot. Both products had the expected u.v.-absorption spectra.

3-Methylcholanthrylene (XIV) (22 mg.), in ethanol (2 ml.), was cooled to −5° and an aqueous solution of $KMnO_4$ (5 mg.), in aq. 5% (w/v) $MgSO_4$ (0·5 ml.), was added over 1·5 hr. The mixture was decolorized with SO_2 and extracted with ether (50 ml.). The ether was evaporated and the residue examined on thin-layer chromatograms developed with solvent (c). A product with the mobility and properties of *cis*-1,2-dihydroxy-3-methylcholanthrene (VII) was detected, together with a small amount of the *trans*-isomer.

3-Methylcholanthrylene (XIV) (100 mg.), in benzene (5 ml.), was treated with silver benzoate (200 mg.) and iodine (98 mg.) according to Hopkins, Brooks & Young (1962) for the preparation of *cis*- and *trans*-1,2-dihydroxyacenaphthene. The benzoates thus obtained formed red gummy solids: they were hydrolysed with KOH in dioxan–methanol and at room temperature as described by Hopkins *et al.* (1962). If the addition reaction was carried out at room temperature, *cis*-1,2-dihydroxy-3-methylcholanthrene (VII) (21 mg.) was formed, separating from aq. ethanol in pale-green needles, m.p. and mixed m.p. 225°, whereas if the reaction was carried out under reflux, *trans*-1,2-dihydroxy-3-methylcholanthrene (VII) (15 mg.) was obtained, separating from aq. ethanol in pale-green needles, m.p. and mixed m.p. 206°. When the benzoate obtained from the reaction carried out at room temperature was hydrolysed with KOH in boiling methanol a product was obtained that, when examined on thin-layer chromatograms, was found to contain compounds with the properties of both the *cis*- and the *trans*-isomers of (VII).

3-Methylcholanthrene-1,2-quinone (IV) (50 mg.), in dry ether (50 ml.), was reduced with lithium tetrahydroaluminate (100 mg.) as described above. The product was examined on thin-layer chromatograms developed with solvent (c) and found to contain a compound with the mobility and properties of *trans*-1,2-dihydroxy-3-methylcholanthrene, together with small amounts of products with the mobilities and properties of the *cis*-dihydroxy compound and of 1- and 2-hydroxy-3-methylcholanthrene (X and VIII).

Oxidation of 3-methylcholanthrylene (XIV) *with perbenzoic acid.* In an attempt to prepare 1,2-epoxy-3-methylcholanthrene, 3-methylcholanthrylene (XIV) (500 mg.) and perbenzoic acid (260 mg.), in chloroform (12 ml.), were kept at 0° for 48 hr. The solid that separated was recrystallized from benzene to yield a *monobenzoate* of *cis*-1,2-dihydroxy-3-methylcholanthrene (220 mg.), separating from benzene in needles, m.p. 213° (Found: C, 82·7; H, 5·1. $C_{28}H_{20}O_3$ requires C, 83·15; H, 5·0%); $\lambda_{max.}$ at 225, 232, 263, 274, 284, 295, 325, 341, 357 and 376 mμ. The infrared spectrum showed strong carbonyl absorption at 1730 cm.$^{-1}$. Hydrolysis of the product with KOH in boiling methanol yielded *cis*-1,2-dihydroxy-3-methylcholanthrene (VII), m.p. and mixed m.p. 225°.

The chloroform mother-liquors were examined on thin-layer chromatograms developed with solvents (b) and (c) and found to contain a product with the mobility and fluorescence of 3-methylcholanthren-2-one (XII). A product with the expected properties of the 1,2-epoxy compound was not detected.

cis-*11,12-Dihydro-11,12-dihydroxy-3-methylcholanthrene* (V). This was prepared from the hydrocarbon (III) by the action of OsO_4 as described by Cook & Schoental (1948), when it formed needles from benzene, m.p. 216–217° (decomp.); $\lambda_{max.}$ at 219, 239, 257, 265, 274, 311 and 348 mμ (log ε 4·69, 4·34, 4·60, 4·73, 4·73, 4·15 and 3·28 respectively). Cook & Schoental (1948) report m.p. about 215° (decomp.). When the dihydrodihydroxy compound (V) was heated with HCl in acetic acid as described by these authors, a phenol was obtained that is probably 11-hydroxy-3-methylcholanthrene (I, R = H). This was difficult to purify, but the methoxy derivative (I, R = Me), prepared by the

action of diazomethane in ether on the phenol and purified by chromatography in benzene on Florisil, crystallized from light petroleum (b.p. 80–100°) in yellow needles, m.p. 203–204°; $\lambda_{max.}$ at 225, 264, 285, 295, 309, 344, 357, 360, 368 and 399 mμ. Cook & Schoental (1948) give m.p. 204–205°. The possibility that the phenol (I, R=H) and its derivative are the 12-isomers is not excluded, but is less likely.

3-*Methylcholanthrene*-11,12-*quinone*. *cis*-11,12-Dihydro-11,12-dihydroxy-3-methylcholanthrene (V) (1g.), in acetic acid (50ml.), was heated to 60° and $Na_2Cr_2O_7$ (5g.) was added and the mixture stirred for 15min. The solid that separated was recrystallized from acetic acid to yield 3-*methylcholanthrene*-11,12-*quinone* (850mg.) in red needles with a metallic lustre, decomp. at 238° (Found: C, 84·4; H, 4·7. $C_{21}H_{14}O_2$ requires C, 84·5; H, 4·7%); $\lambda_{max.}$ at 228, 275 and 310 mμ.

trans-11,12-*Dihydro*-11,12-*dihydroxy*-3-*methylcholanthrene* (V). 3-Methylcholanthrene-11,12-quinone (500mg.), in ether (250mg.), was reduced with lithium tetrahydroaluminate (1g.) and the product isolated as before. trans-11,12-*Dihydro*-11,12-*dihydroxy*-3-*methylcholanthrene* (V) (210mg.) separated from benzene in needles, m.p. 193° (Found: C, 83·0; H, 6·1. $C_{21}H_{18}O_2$ requires C, 83·4; H, 6·0%), $\lambda_{max.}$ at 220, 240, 255, 265, 275, 294, 310 and 351 mμ ($\log \epsilon$ 4·57, 4·28, 4·45, 4·65, 4·69, 4·04, 4·12 and 3·31 respectively). The compound (V) was hydrolysed with HCl in acetic acid and the product methylated with diazomethane and purified as before to yield 11-methoxy-3-methylcholanthrene (I, R=Me) in yellow needles, m.p. and mixed m.p. 201–202°.

11,12-*Epoxy*-11,12-*dihydro*-3-*methylcholanthrene* (II). To a solution of *cis*-11,12-dihydro-11,12-dihydroxy-3-methylcholanthrene (V) (1·55g.) in methanol (2l.) and water (300ml.) was added a solution of $NaIO_4$ (4·3g.) in methanol (400ml.) and water (75ml.). The solution was stirred at room temperature for 24hr. and filtered and the filtrate was evaporated to 250ml. under reduced pressure. The solid that separated was recrystallized from light petroleum (b.p. 80–100°) to yield 3-*methyl*-7-*phenylacenaphthene*-2′,8-*dicarboxaldehyde* (800mg.) in pale-yellow crystals, m.p. 151–152° (Found: C, 83·95; H, 5·4. $C_{21}H_{16}O_2$ requires C, 84·0; H, 5·4%). The dialdehyde (500mg.), in dry benzene (5ml.), was heated under reflux with tri(dimethylamino)-phosphine (0·5ml.), following the method of Newman & Blum (1964). After 15min. the solution was cooled in ice and the product that separated was recrystallized from benzene to yield 11,12-*epoxy*-11,12-*dihydro*-3-*methylcholanthrene* (II) (275mg.) in needles that turned orange at 140°, softened and darkened at 222° and melted at 255–265° (Found: C, 88·5; H, 5·4. $C_{21}H_{16}O$ requires C, 88·7; H, 5·7%); $\lambda_{max.}$ at 260, 269, 280, 302, 313, 324, 342 and 359 mμ ($\log \epsilon$ 4·52, 4·71, 4·76, 4·09, 4·14, 4·11, 3·83 and 3·82 respectively).

The epoxide (II) was heated under reflux with aq. 50% (v/v) acetone (100ml.) containing conc. HCl (1ml.) for 30min. The acetone was distilled off under reduced pressure and the mixture extracted with ether (50ml.). The residue obtained on evaporation of the ether contained a product that had the same mobility and properties on thin-layer chromatograms as the phenol (I, R=H) described above. The methyl ether, prepared and purified as before, had m.p. 200–202°, undepressed in admixture with the methyl ether (I, R=Me).

The epoxide (II) (100mg.), in aq. 50% (v/v) acetone (250ml.), was heated under reflux for 48hr. The acetone was distilled off under reduced pressure and the solid collected and washed with aq. 2N-KOH (100ml.) and recrystallized twice from benzene to yield *trans*-11,12-dihydro-11,12-dihydroxy-3-methylcholanthrene (V) (18mg.), m.p. and mixed m.p. 193°. The u.v. spectrum was identical with that of the *trans*-isomer described above. The aqueous layer was acidified with HCl and extracted with ether. The ether extract was examined on thin-layer chromatograms developed with solvent (b) and found to contain a product with the mobility and fluorescence of the phenol (I, R=H).

S-(11,12-*Dihydro*-12-*hydroxy*-3-*methyl*-11-*cholanthrenyl*)-*glutathione* (IX). The epoxide (II) (50mg.), $NaHCO_3$ (100mg.) and glutathione (50mg.), in aq. 50% (v/v) acetone (200ml.), were heated under reflux for 6hr. The acetone was distilled off under reduced pressure and the mixture extracted with ether (50ml.). The ether extract was examined on thin-layer chromatograms developed in solvent (b) or (c) and shown to contain compounds with the mobilities and properties of the phenol (I, R=H) and the dihydrodihydroxy compound (V). The aqueous layer was acidified to pH4 with acetic acid and activated charcoal (British Drug Houses Ltd., Poole, Dorset) (5g.) was added. The charcoal was filtered off and washed with water (250ml.) and the adsorbed material eluted with methanol (500ml.) containing 5% (v/v) of aq. NH_3 (sp.gr. 0·88). Evaporation of the solvent yielded a gum that is probably a mixture of the ammonium salts of the diastereoisomeric forms of *S*-(11,12-dihydro-12-hydroxy-3-methyl-11-cholanthrenyl)-glutathione (IX), although the possibility of their being the isomeric 11-hydroxy-12-cholanthrenyl derivatives is not excluded. The u.v.-absorption spectrum, measured in methanol, showed $\lambda_{max.}$ at 222, 268, 277, 310 and 352 mμ. On paper chromatograms, the product had R_F 0·10 and 0·45 in solvents 1 and 2 respectively, had a dark-violet fluorescence in u.v. light and gave a purple colour with ninhydrin and a positive reaction with the platinic iodide reagent. The conjugate was decomposed rapidly with conc. HCl to yield three products, one of which had the u.v.-absorption spectrum, the chromatographic mobility in solvents (a) and (b) and the fluorescence of 3-methylcholanthrene (III) on thin-layer chromatograms. Of the other two products, one had R_F 0·18 and 0·61 on paper chromatograms in solvents 1 and 2 respectively, a bright-blue fluorescence in u.v. light and gave a purple colour with ninhydrin and a weak positive reaction with the platinic iodide reagent: it is probably *S*-(3-methyl-11-cholanthrenyl)glutathione. The other product was indistinguishable from oxidized glutathione on paper chromatograms developed with solvent 2: reduced glutathione was not detected.

Experiments with rat-liver homogenates

Young male rats of the Chester Beatty strain (body wt. approx. 180g.) were used in these experiments, each of which was carried out with the livers of four of these rats. The livers were homogenized in 200ml. of ice-cold 1·15% (w/v) KCl in a Potter & Elvehjem (1936) type of homogenizer with a Teflon pestle. The homogenate was centrifuged at 0° for 20min. at 1480$g_{av.}$ in an Angle 50 centrifuge (Measuring and Scientific Equipment Ltd., Birmingham) and the supernatant diluted with an equal amount of 0·1M-phosphate buffer, pH7·4, prepared from NaH_2PO_4 and Na_2HPO_4. Nicotinamide (1·76g.) (Roche Products Ltd.),

$NADP^+$ (30mg.) and glucose 6-phosphate (250mg.) (both as the sodium salts from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany) were added and the mixture was heated in a water bath to 37° while a slow stream of air was drawn through. The substrate (2–10mg., as indicated below), in ethanol (5ml.), was added and the incubation continued for 30min. The mixture was extracted twice with ether (250ml.): if necessary the organic and the aqueous phase were separated by centrifugation. In experiments in which the aqueous phases were not examined, better yields of the metabolites were obtained if the incubation mixtures were first acidified with conc. HCl.

The ether extract was dried over Na_2SO_4 and evaporated and the residue chromatographed on two thin-layer chromatograms that were developed for 15cm. in solvent (b). Fluorescent bands, seen when the chromatograms were examined in u.v. light, were removed and the adsorbed material was eluted with ether. The fractions thus obtained were each separately chromatographed on fresh thin-layer chromatograms in solvent (a), (b) or (c). The R_F values given below are those obtained when the fractions were rechromatographed. The subsequent treatment of the various fractions is described below: usually only one of these investigations could be carried out on the material obtained from one incubation.

The aqueous phase was heated at 100° for a few minutes to coagulate protein and filtered and the filtrate was acidified to pH4 with acetic acid and charcoal (5g.) was added. The charcoal was filtered off and washed with water (250ml.) and the adsorbed material eluted with methanol containing 5% (v/v) of aq. NH_3 (sp.gr. 0·88). The solvent was removed under reduced pressure and the residue examined as described below.

Control experiments were carried out in which the rat-liver homogenates were boiled for 5min. and cooled to room temperature before the addition of the factors described above. The mixtures were then incubated at 37° in the presence of the substrates and the mixture was extracted and the products were examined as before. One incubation was carried out with fresh rat-liver homogenate in the absence of a substrate: none of the metabolites described below was detected.

Experiments were also carried out in which 3-methylcholanthrene (III) was incubated with mouse-liver homogenates: the conditions were the same as in the experiments with rat-liver homogenates.

As far as possible all the operations described above were carried out in the dark or in the absence of direct sunlight to reduce the possibility of photochemical oxidations occurring during the incubations and the preparation procedures.

RESULTS

Metabolism of 3-*methylcholanthrene* (III). In each experiment the hydrocarbon (10mg.) was incubated with rat-liver homogenate as described above. The ether-soluble portion yielded ten fractions by chromatography on thin-layer chromatograms.

Fraction 1 was identified as unchanged 3-methylcholanthrene (III) by its mobility in solvents (a) and (b) and by its u.v.-absorption spectrum.

Fraction 2 sometimes contained an unidentified metabolite, which had R_F 0·49 in solvent (b) and a violet fluorescence in u.v. light. Its u.v.-absorption spectrum showed $\lambda_{max.}$ at 232, 263, 272, 284, 295, 302, 325, 355, 374 and 391mμ.

Fraction 3 consisted of a product with the mobility in solvents (b) and (c) and the fluorescence of 3-methylcholanthren-1-one (XIII), which, on oxidation with sodium dichromate and methylation of the product, yielded a compound indistinguishable from the ester (VI, R = Me) on thin-layer chromatograms. Sometimes the fluorescence in u.v. light was yellow–green, indicating the probable presence of 3-methylcholanthren-2-one (XII) as well as the ketone (XIII).

Fractions 4 and 5 contained small amounts of compounds, R_F 0·37 and 0·32 in solvent (b), both of which had a violet fluorescence in u.v. light that changed to pink and green respectively when the chromatograms were exposed to ammonia. The faster-moving compound gave a blue colour when the chromatograms were sprayed with a 0·5% solution of 2,6-dichloroquinonechloroimide in ethanol followed by aq. 10% (w/v) sodium carbonate. The probable structures of these compounds are discussed below.

Fraction 6, R_F 0·20 and 0·62 in solvents (b) and (c) respectively, was identical in its mobility and fluorescence with 1-hydroxy-3-methylcholanthrene (X), and it had the expected u.v.-absorption spectrum. Oxidation with sodium dichromate and methylation of the product yielded two esters: that present in the larger amount had the mobility and properties of the ester (VI, R = Me) and the other those of the ester (XI, R = Me) on thin-layer chromatograms developed with solvents (b) and (c). The u.v.-absorption spectrum of the former product was identical with that of the ester (VI, R = Me). When the metabolite was allowed to stand in air for some hours and examined on thin-layer chromatograms developed with solvent (b) or (c), an additional spot with the mobility and properties of the ketone (XIII) was detected. When the metabolite was treated with acetic anhydride (0·1ml.) in pyridine (0·1ml.) and the product examined on thin-layer chromatograms, it was found to be converted into a product with the mobility in solvents (b) and (c) of 1-acetoxy-3-methylcholanthrene. The u.v.-absorption spectrum of this product showed the expected maxima.

Fraction 7, R_F 0·18 and 0·82 in solvents (b) and (c) respectively, had an olive-green fluorescence in u.v. light and was indistinguishable from 3-methylcholanthrene-1,2-quinone (IV).

Fraction 8, R_F 0·15 and 0·55 in solvents (b) and (c) respectively, was identical in its mobility and properties on thin-layer chromatograms with 2-hydroxy-3-methylcholanthrene (VIII). It had the expected u.v.-absorption spectrum and, after

oxidation with sodium dichromate and methylation of the product, yielded a compound with the mobility and properties on thin-layer chromatograms developed with solvents (b) and (c) of the ester (XI, R = Me). The u.v.-absorption spectrum was the same as that of the synthetic ester. The metabolite was slowly oxidized in air to give a product that was indistinguishable from the ketone (XII) on thin-layer chromatograms. Acetylation as before yielded a product with the mobility on thin-layer chromatograms developed in solvent (b) or (c) and with the u.v.-absorption spectrum of 2-acetoxy-3-methylcholanthrene.

Fraction 9, R_F 0·10 in solvent (b), had a violet fluorescence in u.v. light and its u.v.-absorption spectrum showed $\lambda_{max.}$ at 223, 236, 263, 274, 285, 295, 325, 341, 357 and 375mμ. When the metabolite was treated with acetic anhydride in pyridine, it was converted into a product, R_F 0·49 in solvent (b), whose u.v. spectrum showed the same maxima as that of the parent metabolite. Oxidation of the metabolite with sodium dichromate and methylation of the product did not yield any recognizable products. A possible structure for the metabolite is discussed below.

Fraction 10, which consisted of the material left at the base lines of the original chromatograms, was examined on thin-layer chromatograms developed with solvent (c). Four major products were detected, together with small amounts of several unidentified fluorescent substances. The first metabolite, R_F 0·28, had the chromatographic properties of *cis*-1,2-dihydroxy-3-methylcholanthrene (VII), the second, R_F 0·23, had the properties of 11,12-dihydro-11,12-dihydroxy-3-methylcholanthrene (V), and the third, R_F 0·20, the properties of *trans*-1,2-dihydroxy-3-methylcholanthrene (VII). The fourth product had R_F 0·16.

On the two-dimensional chromatograms described above, it was shown that the metebolites with the mobilities of the isomeric dihydroxy compounds (VII) were each converted into a product with the chromatographic mobility in solvent (b) and the properties of 3-methylcholanthren-2-one (XII). The ketone derived from the *cis*-isomer formed a larger spot on the chromatogram than that derived from the *trans*-isomer, indicating that the *cis*-isomer was the major product. By combining the products from a number of incubations, it was shown that the u.v.-absorption spectrum of the ketone derived from the *cis*-isomer was identical with that of the synthetic product (XII). It was also shown that, when this decomposition product was oxidized with sodium dichromate and the product methylated, a compound with the chromatographic mobility in solvents (b) and (c) of the ester (XI, R = Me) was formed. When the mixture of metabolites in fraction 10 was acetylated as before, products with the mobilities and properties on thin-layer chromatograms of *cis*- and *trans*-1,2-diacetoxy-3-methylcholanthrene were detected.

On these two-dimensional chromatograms, the product of R_F 0·23 was decomposed to yield a phenol with the mobility in solvent (b) and the fluorescence in u.v. light of 11-hydroxy-3-methylcholanthrene (I, R = H), thus providing additional evidence for the presence of the parent compound (V) in the mixture of metabolites.

The product of R_F 0·16 was shown on these two-dimensional chromatograms to yield two products with acid, R_F 0·46 and 0·27 respectively, that appeared to be phenolic in character: both products formed spots with a violet fluorescence in u.v. light, which changed to light-blue and blue respectively when the chromatograms were exposed to ammonia. Neither the phenol nor the parent compound, which appeared to be a dihydrodihydroxy compound, was identified.

The gummy material obtained from the aqueous portion as described above was applied to the base line of a paper chromatogram, which was developed with solvent 1. The paper was dried and examined in u.v. light and the dark-violet fluorescent band at about R_F 0·10 was cut out and the adsorbed material eluted with methanol containing 1% (v/v) of aq. ammonia (sp.gr. 0·88). The solvent was evaporated and the small residue rechromatographed on paper and the product located and eluted as before. The u.v.-absorption spectrum of the material thus obtained showed $\lambda_{max.}$ at 222, 268, 277, 310 and 352mμ. The product was indistinguishable in its chromatographic mobility in solvents 1 and 2, in its fluorescence in u.v. light and in its reaction with ninhydrin and the platinic iodide reagent from the synthetic glutathione conjugate (IX) described above. Three products were formed when the conjugate was acidified with conc. hydrochloric acid. One was identical in its mobility on paper chromatograms in solvents 1 and 2, in its bright-blue fluorescence in u.v. light and in its reaction with ninhydrin and the platinic iodide reagent with the acid-decomposition product of the conjugate (IX) described above. The second product was indistinguishable on paper chromatograms developed with solvent 2 from oxidized glutathione. The third decomposition product was indistinguishable in its u.v. spectrum and in its mobility on thin-layer chromatograms developed in solvents (a) and (b) from 3-methylcholanthrene (III).

None of the products described above was detected in experiments using heated homogenates.

In the experiments with mouse-liver homogenates, metabolites with the chromatographic properties of 1- and 2-hydroxy-3-methylcholanthrene (X and VIII), 3-methylcholanthren-1-one (XIII),

3-methylcholanthrene-1,2-quinone (IV), *cis-* and *trans-*1,2-dihydroxy-3-methylcholanthrene (VII), 11,12-dihydro-11,12-dihydroxy-3-methylcholanthrene (V) and the unidentified product of R_F 0·10 in solvent (b) were detected. The unidentified phenols and dihydrodihydroxy compound were also found and the aqueous fraction was not examined.

Metabolism of 1- *and* 2-*hydroxy*-3-*methylcholanthrene* (X *and* VIII). Samples (2mg.) of either (X) or (VIII) were used in each experiment, and only the ether-soluble material was examined.

1-Hydroxy-3-methylcholanthrene (X) was converted into a product with the mobility on thin-layer chromatograms developed in solvents (b) and (c), the fluorescence in u.v. light, and the u.v.-absorption spectrum of 3-methylcholanthren-1-one (XIII). Oxidation of this metabolite with sodium dichromate and methylation of the product yielded a compound with the chromatographic mobility in solvents (b) and (c) of the ester (VI, R=Me). 2-Hydroxy-3-methylcholanthrene (VIII) yielded 3-methylcholanthren-2-one (XII), which was similarly identified. A product with the mobility of the ester (XI, R=Me) on thin-layer chromatograms was obtained when the metabolite was oxidized and the product methylated as before.

Both hydroxy compounds (X and VIII) also yielded mixtures of *cis-* and *trans-*1,2-dihydroxy-3-methylcholanthrene (VII), which were identified in the same way as those obtained in the metabolism of the parent hydrocarbon (III). In both cases, more *cis-* than *trans-*isomer appeared to have been formed, as judged from the sizes and the intensities of the spots of the ketone (XII) produced when the products were examined on two-dimensional thin-layer chromatograms as described above.

Both hydroxy compounds yielded a third metabolite, which had R_F 0·01 in solvent (c) and was detected by its violet fluorescence in u.v. light. It was converted by hot conc. hydrochloric acid into a product that had R_F 0·05 in solvent (b) and 0·15 in solvent (c) and an orange–yellow fluorescence in u.v. light.

Products were sometimes detected in these experiments that resembled the unidentified products, R_F 0·49 in solvent (b), detected in the experiments with the hydrocarbon (III), in their mobilities, their fluorescence in u.v. light and their u.v.-absorption spectra. They were not identified.

In experiments with heated homogenates, the hydroxy compounds (X and VIII) yielded the related ketones (XIII and XII), which were identified in the manner already described. The other products formed in the enzymic experiments were not detected.

Metabolism of 3-*methylcholanthren*-1- *and* -2-*one* (XIII *and* XII). Experiments were carried out on 2mg. samples of these compounds, and, in both cases, the unchanged ketones were detected. Both ketones yielded products that were identified as the respective hydroxy compounds (X) and (VIII) by their mobilities on thin-layer chromatograms in solvents (b) and (c), by their u.v.-absorption spectra and by the mobilities of the methyl esters obtained from the products of the oxidations with sodium dichromate, that from the ketone (XIII) yielding mainly the ester (VI, R=Me) and that from the ketone (XII) yielding only the ester (XI, R=Me).

Both ketones were converted by the homogenates into a mixture of *cis-* and *trans-*1,2-dihydroxy-3-methylcholanthrene (VII), which were identified on two-dimensional thin-layer chromatograms as before. The *cis-*isomer appeared to be the major product.

3-Methylcholanthren-1-one (XIII) yielded a product that had R_F 0·05 and 0·10 in solvents (b) and (c) respectively and a bright-green fluorescence in u.v. light. 3-Methylcholanthren-2-one (XII), on the other hand, yielded a product which had the same R_F values as that formed from the 1-isomer, but which had an orange–yellow fluorescence: it resembled the product detected after the acid-decomposition of the slow-moving metabolites of the hydroxy compounds (X and VIII). Possible structures for these products are discussed below.

Metabolites with the properties of the unidentified 3-methylcholanthrene metabolite of R_F 0·49 in solvent (*b*) were sometimes detected in these experiments.

Metabolism of cis- *and* trans-1,2-*dihydroxy*-3-*methylcholanthrene* (VII). Experiments were carried out on 5mg. samples of the isomers, and in both cases products with the mobilities in solvent (b) and the u.v.-absorption spectra of the unidentified 3-methylcholanthrene metabolite of R_F 0·49 were detected.

Although large amounts of the substrates were unchanged in these incubations, some conversions of the *cis-* into the *trans-* and of the *trans-* into the *cis-*isomer occurred, since small spots with the chromatographic mobilities of the second isomers were always detected. These were further identified by their decomposition on two-dimensional thin-layer chromatograms by the formation of products indistinguishable from the ketone (XII). These isomerizations were apparently enzymic in nature because they did not occur either when heated homogenates were used or when the dihydroxy compounds (VII) were each heated at 50° for 2hr. with 0·1M-phosphate buffer.

The *cis-* and the *trans-*dihydroxy compounds (VII) were both converted by homogenates into a product with the mobility on thin-layer chromatograms and the fluorescence in u.v. light of the slow-running metabolite of the hydroxy compounds

(X and VIII), and these were both decomposed by acid into a product with the mobility and fluorescence of the slow-running metabolite of 3-methylcholanthren-2-one (XII).

Occasionally, a product with the mobility in solvents (b) and (c) and the fluorescence of 3-methylcholanthrene-1,2-quinone (IV) was detected in these incubations: this product was also detected in the experiments with heated homogenates.

Metabolism of 3-methylcholanthrylene (XIV). In these experiments, which were carried out on 10mg. samples, six fractions were detected in the ether-soluble portion and separated as before. Fraction 1 was identified as 3-methylcholanthren-2-one (XII) by its mobility in solvents (b) and (c), by its fluorescence in u.v. light and by its u.v.-absorption spectrum. Fraction 2 was indistinguishable from the quinone (IV) in its mobility in solvents (a) and (b) and in its fluorescence. Fraction 3 was identified by its mobility in solvents (b) and (c), by its fluorescence and by its u.v.-absorption spectrum as 2-hydroxy-3-methylcholanthrene (VIII). The ester obtained when the product of the oxidation of this metabolite with sodium dichromate was methylated was indistinguishable on thin-layer chromatograms from the ester (XI, R = Me).

Fractions 4 and 5 contained metabolites indistinguishable from *cis*- and *trans*-1,2-dihydroxy-3-methylcholanthrene (VII): these were identified by their chromatographic mobilities in solvent (c) and by their conversion into products indistinguishable from the ketone (XII) on two-dimensional thin-layer chromatograms. From an inspection of these chromatograms it was evident that the *trans*-compound was the major product.

A metabolite in fraction 6 was identical in its mobility in solvents (b) and (c) and in its fluorescence with the slow-running metabolite of 3-methylcholanthren-2-one (XII).

The aqueous fraction contained a product that had R_F 0·15 on paper chromatograms developed with solvent 1. It was detected by its dark absorption when the chromatograms were examined in u.v. light, and it gave a purple colour with ninhydrin and a positive reaction with the platinic iodide reagent. The product was not further investigated but it is presumed to be *S*-(2-hydroxy-3-methyl-1-cholanthrenyl)glutathione or possibly the isomeric 1-hydroxy-2-cholanthrenyl derivative.

Metabolism of 11,12-epoxy-11,12-dihydro-3-methylcholanthrene (II). The epoxide (II) (10mg.) was incubated with rat-liver homogenate as before, and both the ether-soluble and the aqueous fractions were examined. The ether-soluble fraction contained two products, the first of which was identified by its mobility in solvents (b) and (c), its fluorescence and its u.v.-absorption spectrum as the phenol that probably has the structure (I). The second product had the mobility of 11,12-dihydro-11,12-dihydroxy-3-methylcholanthrene (V) in solvent (c); additional evidence for its structure was provided by the fact that on two-dimensional thin-layer chromatograms it was decomposed with acid to a product with the mobility and fluorescence of the phenol (I).

The aqueous phase contained a compound with the mobility in solvents 1 and 2 on paper chromatograms of the glutathione conjugate (IX). It was detected as a dark-violet fluorescent spot when the chromatograms were examined in u.v. light and it gave a purple colour with ninhydrin and a positive reaction with the platinic iodide reagent. When the conjugate was acidified with conc. hydrochloric acid, it yielded products with the chromatographic properties of those previously detected in the acid-decomposition of the synthetic conjugate and of that found in the metabolism of the hydrocarbon (III).

In experiments with heated homogenates, products with the chromatographic properties of the epoxide (II) together with relatively small amounts of compounds with the properties of the phenol (I) and the dihydrodihydroxy compound (V) were detected on thin-layer chromatograms. The glutathione conjugate (IX) was not found in the aqueous fraction.

DISCUSSION

The acid (VI, R = H) can be decarboxylated to 8,9-dimethylbenz[*a*]anthracene-7,12-quinone, the structure of which was proved by an unambiguous synthesis (Cook & Haslewood, 1934). Since 1-hydroxy-3-methylcholanthrene (X) and the related ketone (XIII) both yield mainly this acid in the chromic acid oxidation of the hydrocarbon (III) the positions of the hydroxy and oxo groups at C-1 on the molecule are established. The structure of the second oxidation product of the hydrocarbon (III) has not been proved unambiguously, but an acid with the proposed structure (XI, R = H) would be expected if, in the minor oxidative pathway, the primary attack of the oxidizing agent was at C-2 on the hydrocarbon (III). The u.v.-absorption spectrum of the related methyl ester (XI, R = Me) is that expected for a derivative of benz[*a*]anthracene-7,12-quinone.

The position of a hydroxyl group at C-1 of the isomeric dihydroxy compounds (VII) is shown by their syntheses from 1-acetoxy-3-methylcholanthrene. Since only the acid (XI, R = H) was formed in the chromic acid oxidations of these compounds, the second hydroxyl group is probably at C-2, and this is confirmed by the existence of *cis*- and *trans*-isomers. The light-absorption curves are those expected for hydroxy derivatives of 3-methylcholanthrene (III) that are not phenolic.

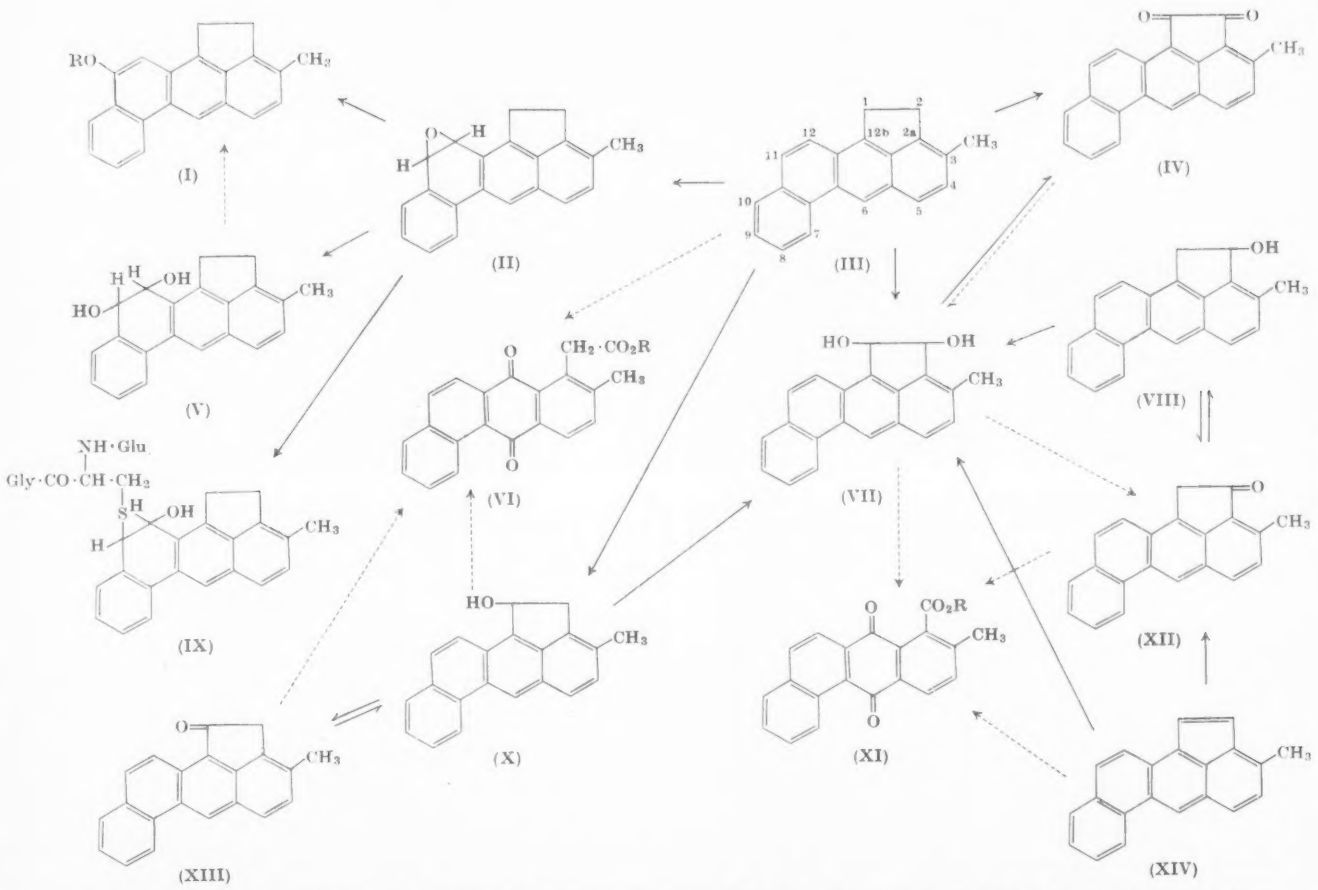
The formation of 3-methylcholanthrylene (XIV) in the lead tetra-acetate oxidation of the hydrocarbon (III) is unexpected and it probably arises from 1-acetoxy-3-methylcholanthrene by loss of acetic acid, probably during the column chromatography. 1-Acetoxyacenaphthene is converted into acenaphthylene on passing through a heated tube (Flowers & Miller, 1947). The bromination of the hydrocarbon (III) presumably leads to the formation of the 1-bromo derivative, which loses hydrogen bromide across the 1,2-positions to yield the hydrocarbon (XIV). The formation of only the acid (XI, R = H) in the chromic acid oxidation of the hydrocarbon (XIV) is additional evidence for its structure.

3-Methylcholanthrene-1,2-quinone (IV) probably arises by oxidation of the isomeric dihydroxy compounds (VII), either during the alkaline hydrolysis of their acetoxy derivatives or on the Florisil column. The formation of the isomeric dihydroxy compounds (VII) in the reduction of the diketone (IV) with lithium tetrahydroaluminate provides evidence for this structure. The reduction also provides confirmatory evidence that the assignment of the *cis*- or *trans*-configuration to the isomers of (VII) on the basis of their reaction with the potassium triacetylosmate reagent is correct, since the isomer believed to have the *trans*-configuration is the major product of the reduction. Acenaphthene-1,2-quinone is similarly reduced to a mixture containing 15% of the *cis*- and 45% of the *trans*-isomer of 1,2-dihydroxyacenaphthene (Trevoy & Brown, 1949). On the other hand, the permanganate oxidation of 3-methylcholanthrylene (XIV) yielded mainly the product believed to be the *cis*-isomer of (VII): the oxidation of olefins with permanganate usually gives *cis*-dihydroxy compounds. The reactions of the hydrocarbon (XIV) with silver benzoate and iodine similarly yielded the expected isomers.

The dehydration of the 1,2-dihydroxy compounds (VII) with acid yields only one ketone, which is not 3-methylcholanthren-1-one (XIII): it must therefore be the 2-isomer (XII). The product obtained from this on reduction with lithium tetrahydroaluminate is therefore 2-hydroxy-3-methylcholanthrene (VIII). *cis*- and *trans*-1,2-Dihydroxyacenaphthene are dehydrated with acid to acenaphthen-1-one (Hopkins *et al.* 1962). The ketone (VII) and the hydroxy compound (VIII) both yielded the expected acid (XI, R = H) on oxidation with chromic acid, showing that the oxo and the hydroxy groups are at C-2 on the nucleus. Additional evidence for the formulation of the hydroxy compound as (VIII) is provided by the fact that the oxidation of the related acetoxy derivative with lead tetra-acetate leads to the formation of the isomers of 1,2-dihydroxy-3-methylcholanthrene (VII).

11,12-Epoxy-11,12-dihydro-3-methylcholanthrene (II) has properties similar to those of the epoxides related to phenanthrene, benz[*a*]anthracene, 7-methylbenz[*a*]anthracene and dibenz[*a*,*h*]anthracene (Newman & Blum, 1964; Boyland & Sims, 1965*b*). It readily rearranges to the phenol (I, R = H), reacts with water to give the *trans*-isomer of the dihydrodihydroxy compound (V) and with glutathione to give the glutathione conjugate (IX). The phenol (I, R = H) and the glutathione conjugate (IX) have been formulated as shown in Scheme 1 by analogy with the structures of the products derived from the other epoxides mentioned above. The decomposition of the conjugate (IX) with acid to yield mainly the parent hydrocarbons is also analogous to the decomposition of conjugates derived from the other epoxides.

It is evident that most of the metabolic activity of 3-methylcholanthrene (III) is centred about the 1- and 2-positions and at the 11,12-bond. The small amounts of the phenols detected in fractions 4 and 5 in the experiments with rat- and mouse-liver homogenates are similar in their properties on thin-layer chromatograms to 4- and 3-hydroxybenz[*a*]anthracene (Boyland & Sims, 1964) and are therefore probably 10- and 9-hydroxy-3-methylcholanthrene respectively. The 10-isomer is possibly that detected by Harper (1959) in the metabolism of 3-methylcholanthrene (III) in mice. The product in fraction 10, which was decomposed by acid to give two unidentified phenols, could, by analogy with benz[*a*]anthracene metabolism, be 4,5-dihydro-4,5-dihydroxy-3-methylcholanthrene, but there is no direct evidence that this is so. The u.v. spectrum of the unidentified metabolite in fraction 9 was similar to that of the parent hydrocarbon (III) and to those of 1- and 2-hydroxy-3-methylcholanthrene (X and VIII) and the metabolite is apparently a hydroxy compound since it reacted with acetic anhydride. It is probable that the metabolite is 3-hydroxymethylcholanthrene: such a metabolite would be expected by analogy with the hydroxylation of the methyl groups of 7,12-dimethylbenz[*a*]anthracene in rat-liver homogenates (Boyland & Sims, 1965*a*). It is also possible that the carboxylic acid detected by Harper (1959) was derived from the metabolite by further oxidation of the hydroxymethyl group. The fact that this and the other unidentified metabolites were not formed when 1- or 2-substituted 3-methylcholanthrene derivatives or the epoxide (II) were incubated with rat-liver homogenates provides further evidence that their formation has involved attack at other positions on the hydrocarbon molecule. The unidentified metabolite in fraction 2, on the other hand, is also formed in the metabolism of 1,2-substituted derivatives of the hydrocarbon (III). The attempted identification of this metabolite was made more



Scheme 1. Reactions of compounds related to 3-methylcholanthrene. Solid arrows indicate reactions occurring in rat-liver homogenate and sometimes chemically: broken arrows indicate chemical reactions only.

difficult by the fact that it was not always formed in the incubations: the reason for this is not apparent.

1- and 2-Hydroxy-3-methylcholanthrene (X and VIII) were clearly formed from the hydrocarbon (III) by an enzymic process, but the related ketones (XIII and XII) could have arisen by the chemical oxidation of the hydroxy compounds during the incubations since they were formed from the respective hydroxy compounds when heated homogenates were used. Similarly the diketone (IV) could have arisen from the dihydroxy compounds (VII) by chemical oxidation. The ketones (XIII and XII) were themselves reduced to their respective hydroxy compounds (X and VIII) by rat-liver homogenates.

The formation of the isomeric 1,2-dihydroxy compounds (VII) is perhaps unexpected since with most substrates only one hydroxyl group is introduced enzymically. Thus, for example, 7,12-dimethylbenz[*a*]anthracene is hydroxylated either on the C-7 or on the C-12 methyl group, but not on both (Boyland & Sims, 1965*a*). Most dihydrodihydroxy compounds derived from aromatic hydrocarbons arise from the hydroxylation of double bonds by a mechanism that probably involves epoxide formation. Hydroxylation at C-1 and C-2 of acenaphthene has not been reported, but Chang & Young (1943) have shown that in whole animals these positions are oxidized to yield 1,8-naphthalic anhydride. With 3-methylcholanthrene (III), the hydroxyl groups are probably introduced in two stages since 1- and 2-hydroxy-3-methylcholanthrene (X and VIII) are themselves hydroxylated by rat-liver homogenates. Although it might be expected that random hydroxylations of this type would yield equal amounts of the two isomers, an inspection of the chromatograms showed quite clearly that the *cis*-isomer was the major product. In this respect the enzymic hydroxylation of the hydrocarbon (III) differed from its oxidation by lead tetra-acetate, where, both chromatographically and by isolation of the products, it was shown that the *trans*-isomer was the major product: it is possible that isomerization of the *cis*- to the *trans*-isomer occurred during the alkaline hydrolysis of the acetates. Similar relationships exist between the enzymic hydroxylations of 1- and 2-hydroxy-3-methylcholanthrene (X and VIII) and the oxidations of their acetates with lead tetra-acetate. The reason for these differences is not clear since, in the metabolism of the hydroxy compounds in rat-liver homogenates, both isomers behave in the same way with respect to their further metabolism.

The isomerizations of *cis*- and *trans*-1,2-dihydroxy-3-methylcholanthrene (VII) by rat-liver homogenates resemble those of *cis*- and *trans*-1,2-dihydroxyacenaphthene and *cis*- and *trans*-1,2-dihydroxyindane, all of which are partly converted into the related isomer when injected into rats (Hopkins, Lewis & Young, 1964). Like the hydroxy compounds (VII), these isomers are stable when incubated by themselves in phosphate buffer. The mechanisms involved in these isomerizations are not known: possibly one of the hydroxy groups is oxidized to an oxo group, which is then reduced back to the hydroxyl. It is significant that these isomerizations have not been observed with dihydrodihydroxy compounds formed by the hydroxylation of aromatic double bonds; in these cases the ketones are tautomeric with, and should readily rearrange to, the corresponding dihydroxy compounds, which are known metabolites of the dihydrodihydroxy compounds.

In the metabolism of 3-methylcholanthrylene (XIV), the *trans*-isomer of 1,2-dihydroxy-3-methylcholanthrene (VII) is the predominant isomer formed. Clearly a different mechanism is in operation here, probably involving the formation of an epoxide at the 1,2-double bond. The glutathione conjugate detected in the metabolism of the hydrocarbon (XIV) presumably arises from the reaction of an epoxide of this type with glutathione, although the possibility of a reaction occurring on the K region is not excluded. 3-Methylcholanthren-2-one (XII), which is also formed in the metabolism of the hydrocarbon (XIV), probably arises either by rearrangement of 2-hydroxy-3-methylcholanthrylene, formed by monohydroxylation at C-2 or by rearrangement of the 1,2-epoxide. It is possible that an isomerization of the 1,2-epoxide to the 2-ketone (XII) occurred during the chemical oxidation of the hydrocarbon (XIV) with perbenzoic acid: the formation of the monobenzoate of the *cis*-1,2-dihydroxy compound (VII) is not unexpected (Swern, 1953). Ketones do not appear to be metabolites of indene (Brooks & Young, 1956) or of acenaphthylene (Hopkins *et al.* 1962) in whole animals, although *cis*- and *trans*-dihydrodihydroxy derivatives are formed from both hydrocarbons.

It is probable that the slow-running metabolites of the hydroxy compounds (X and VIII) and of the isomeric dihydroxy compounds (VII) are the same compound, possibly 1,2-dihydroxy-3-hydroxymethylcholanthrene. The product formed from this compound with acid should, by analogy with the decomposition of the dihydroxy compounds (VII), be 3-hydroxymethylcholanthren-2-one, which might also be expected to arise by the hydroxylation of the methyl group in 3-methylcholanthren-2-one (XII). In fact, a compound with the chromatographic properties of the acid-decomposition product is formed as a metabolite of the ketone (XII). 3-Methylcholanthren-1-one (XIII), on the other hand, forms a different metabolic product, which is probably 3-hydroxymethylcholanthren-1-one. Compounds with the suggested structures

have not been synthesized. These metabolites were not detected as products of the metabolism of 3-methylcholanthrene (III) itself, possibly because smaller amounts were formed.

The metabolic products of 3-methylcholanthrene (III) formed at the K region are similar to those reported for other hydrocarbons, such as benz[*a*]anthracene and dibenz[*a*,*h*]anthracene (Boyland & Sims, 1965*b*). The configuration of the dihydrodihydroxy compound (V) is presumed to be *trans* by analogy with the related products formed in the metabolism of these hydrocarbons. The formation of (V) and of the glutathione conjugate (IX), both from the hydrocarbon (III) and from the epoxide (II), suggests that, as with other hydrocarbons, the formation of an epoxide, in this case (II), is the initial metabolic step at the K region. The absence of the phenol (I, R = H), which could have arisen by hydroxylation of the K region of the hydrocarbon (III), is also in agreement with earlier observations that phenols of this type are not formed in the metabolism of aromatic hydrocarbons. The formation of the phenol (I, R = H) in the metabolism of the epoxide (II) is, however, in agreement with observations on the metabolism of other epoxides of this type: a probable explanation for this has been given (Boyland & Sims, 1965*b*).

There was no evidence for any metabolic action at the 6- or 12b-positions (the L region) of 3-methylcholanthrene (III). Chemically these positions are active, as indicated by the chromic acid oxidations described above, by the oxidation with perbenzoic acid (P. Sims, unpublished work) and by ozonolysis (Moriconi & Taranko, 1963), all of which yielded the acid (VI, R = H). With ozone there was also evidence for attack, to a smaller extent, at the K region and at the 2a,3- and 4,5-bonds. In the ascorbic acid–Fe^{2+}–oxygen system, the only identified products were 1- and 2-hydroxy-3-methylcholanthrene (X and VIII), their related ketones (XIII and XII) and the quinone (VI) (P. Sims, unpublished work).

The relationship between the metabolic products of the hydrocarbon (III) and its carcinogenic properties is not clear. Both 1-hydroxy-3-methylcholanthrene (X) and the related ketone (XIII) have carcinogenic properties when injected subcutaneously into mice (Shear & Leiter, 1941; Badger *et al.* 1940) and tumours have been obtained in mice injected subcutaneously with the epoxide (II) (E. Boyland & P. Sims, unpublished work): other metabolites have not been tested.

I thank Professor E. Boyland for his interest and Miss S. Gowers and Mr T. Rabbitts for skilled technical assistance. This investigation has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the Medical Research Council and the British Empire Cancer Campaign for Research, and by the Public Health Service Research Grant no. CA-03188-08 from the National Cancer Institute, U.S. Public Health Service.

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Biochem. J. (1966) **98**, 229

Fractionation of Deoxyribonucleic Acid from Phage-Infected Bacteria

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(*Received* 15 *April* 1965)

1. DNA has been isolated in 90% yield from T5-infected cultures of *Escherichia coli* 'pulse'-labelled with [^{3}H]thymidine. It had a buoyant density in caesium chloride solution identical with the DNA of mature T5 phage, and no components of unusual buoyant density were detected. 2. The DNA preparation was resolved into two major components of differing specific activity on a column of kieselguhr coated with methylated serum albumin. The DNA of high specific activity could be eluted from the column only with 2N-ammonia, and the firm binding did not appear to be due to an artifact of preparation. 3. A similar fractionation into two DNA components of differing specific activity was observed when the 'pulse'-labelled culture was lysed with sodium dodecyl sulphate and the lysate rocked with phenol. The DNA of high specific activity was found in the interface precipitate between the phenol and aqueous layers. 4. The amounts of DNA in the two fractions were measured at different times after infection and the radioactivity content of each was determined at various times after a short 'pulse' of [^{3}H]-thymidine. The interface fraction contained the replicating phage DNA, and the DNA from mature phage particles appeared in the aqueous fraction. 5. Analogous results were obtained with T2-infected *E. coli*. In the presence of chloramphenicol the DNA in the interface fraction was not converted into DNA extractable into the aqueous layer. Since chloramphenicol prevents the condensation of DNA into phage heads, it is suggested that any DNA in extended configuration is trapped inside the rigid-layer framework of the cell wall. 6. Treatment with lysozyme released much of the DNA from the interface precipitate. This DNA was firmly bound by the chromatographic column and had the same buoyant density in caesium chloride solution as normal T5-phage DNA. Sucrose-gradient sedimentation studies showed that it was heterogeneous and that as much as 60% sedimented faster than T5-phage DNA.

Although many facts are known about the replication of DNA in biological systems, the only example where the structure of the replicating molecule is known is the chromosome of *Escherichia coli* (Cairns, 1963). We decided to investigate the structure of replicating DNA from phage-infected bacteria because phage DNA is smaller than unbroken bacterial DNA and therefore the replicating DNA may be easier to extract in an unbroken state from the bacteria. As well, phage-infected bacteria are capable of rapid and extensive DNA synthesis.

At any given time after infection with phage, bacteria are likely to contain bacterial DNA, replicating phage DNA, and DNA in incomplete and completed intracellular phage particles (Hershey, 1953; Hershey & Melechen, 1957; Koch & Hershey, 1959; Kellenberger, 1959). The problem of contamination of DNA preparations with bacterial DNA can be largely surmounted by using T5-infected *E. coli*, since in this system the bacterial DNA is rapidly and extensively degraded after infection (Pfefferkorn & Amos, 1958; Crawford, 1959). The experiments in the present paper describe the fractionation of DNA from T5-infected *E. coli* as a first stage in studying the molecular size and configuration of the replicating DNA.

MATERIALS AND METHODS

Bacteriophage. Phages T2r$^+$ and T5 were prepared from stocks by lysis of bacteria in liquid media as described by Lunt, Siebke & Burton (1964) and Stone & Burton (1962)

respectively. The preparations were assayed by the double-layer technique (Adams, 1950) by using *E. coli* B with phage T2 and *E. coli* B/2 with phage T5. The latter strain, like strain F (Lanni, 1958), gives a higher yield of T5 plaques than does strain B. The purity of each stock was checked by plating the phage on resistant bacteria (B/2 and B/5). The ^{3}H-labelled T5 phage was prepared as above by adding [^{3}H]thymidine (final concn. 2μc/ml.) to the culture at intervals from 25min. after infection. The ^{32}P-labelled T5 phage was prepared by using a tris–glucose synthetic medium (Hershey, 1955) for growth of the bacteria, and immediately after infection [^{32}P]phosphate (final concn. 33μc/ml.) was added. After lysis the bacteriophage was purified as described by Burgi (1963).

Bacteria. *E. coli* strains B, B/2 and B/5 were kept at 3° on nutrient agar slopes. Cultures were grown at 37° with aeration in a liquid synthetic medium with mannitol as the carbon source (Stone & Burton, 1962). A 15hr. culture was added to 10vol. of fresh medium and aerated. When the cell density reached 4×10^8–5×10^8/ml., the bacteria were infected by adding 10–15 T5 particles/bacterium or five T2 particles/bacterium. In experiments with T5 phage, *E. coli* B/2 was used and immediately before infection the culture was made 1mM with respect to $CaCl_2$. It was important for reproducibility that aeration of T5-infected bacterial cultures was never interrupted.

DNA preparations. DNA was prepared from a suspension of purified T5 phage (2×10^{12}/ml.) by a modification (Davison & Freifelder, 1962) of the phenol method of Mandell & Hershey (1960). DNA was prepared from *Micrococcus lysodeikticus* according to the method of Marmur (1961) and was a gift from Dr G. B. Petersen.

Radioactive materials. [^{3}H]Thymidine nominally labelled in the 6 position and of specific activity 8·6c/m-mole was obtained from The Radiochemical Centre, Amersham, Bucks. Carrier-free [^{32}P]phosphate was obtained from the Oak Ridge National Laboratory, Tenn., U.S.A. Before use the solution was heated at 100° for 10min. and neutralized to convert the pyrophosphate form.

Enzymes. Ribonuclease was purchased from the Sigma Chemical Co. (St Louis, Mo., U.S.A.) as a 5× crystallized material from bovine pancreas. Crystalline deoxyribonuclease was obtained from the Worthington Biochemical Corp. (Freehold, N.J., U.S.A.). Lysozyme and papain were obtained from the British Drug Houses Ltd. (Poole, Dorset) and from the Sigma Chemical Co.

Buffers. Tris buffer solutions were prepared by mixing N-HCl and M-tris to obtain the desired pH; phosphate buffers were prepared from 0·2M-KH_2PO_4 and 0·2M-Na_2HPO_4 and by mixing 0·2M-Na_2HPO_4 with N-NaOH. All pH measurements were made at room temperature with a glass electrode.

Other materials. Phenol was AnalaR grade from British Drug Houses Ltd. and was redistilled before use. Liquefied phenol for chromatography (Fisher Scientific Co., Fairlawn, N.J., U.S.A.) was also used without further purification. Ether was 'anaesthetic' grade from Harrington Brothers (London) and was not usually redistilled before use. The product of the J. T. Baker Chemical Co. (Philadelphia, Pa., U.S.A.) was also used, but after redistillation. Caesium chloride was a product of British Drug Houses Ltd. and was designated 'for ultracentrifuge work'. The product of the Kawecki Chemical Co. (New York, N.Y., U.S.A.) was also used. Sodium dodecyl sulphate was obtained from L. Light and Co. Ltd. (Colnbrook, Bucks.), and also from Bios Laboratories (New York, N.Y., U.S.A.). Chloramphenicol was a product of Parke, Davis and Co. Ltd. (Hounslow, Middlesex). Thymidine was obtained from the California Corp. for Biochemical Research (Los Angeles, Calif., U.S.A.).

'Pulse'-labelling of cultures. Cultures of infected bacteria were 'pulse'-labelled by the addition of [^{3}H]thymidine (final concn. 0·1–0·2μc/ml., except when DNA was required for zone centrifugation, when 7μc/ml. was used) and allowing aeration to continue for 30sec. (a 1min. 'pulse' was used in the preparation for zone centrifugation) before either pouring the culture on to ice or adding a several thousand-fold excess of unlabelled thymidine.

Preparation of lysates. Cultures of infected bacteria were poured on to 0·5vol. of crushed frozen 0·1M-NaCl. The mixture was stirred and centrifuged at 2500*g* for 10min. and the pellet was washed by suspending in ice-cold 0·1M-NaCl and centrifuging. The resultant pellet was suspended evenly in 0·08M-tris buffer, pH8·0, containing EDTA (0·05M), to make a bacterial concentration of 5×10^9–8×10^9 cells/ml. Lysates were prepared by either of the following methods.

(*a*) Lysozyme was added to the suspension to make a final concentration of 300μg./ml. The mixture was incubated at 37° for 5min. and the resultant viscous lysate frozen and thawed three times with the use of an ethanol–solid CO_2 bath. Sodium dodecyl sulphate was then added (final concn. 0·75%, w/v) and the mixture incubated for a further 5min. at 37°. The resultant solution was usually clear.

(*b*) Sodium dodecyl sulphate was added to the washed suspension of infected bacteria to make a final concentration of 0·75% (w/v) and the mixture was incubated at 37° for 5min. The resultant lysate was very viscous but still turbid. In the experiments with phage T2r$^+$, samples of the culture were added directly to a mixture of KCN and sodium dodecyl sulphate (final concn. 10mM-KCN and 0·06% dodecyl sulphate). The mixture was incubated at 37° for 5min. and cooled in ice. This modification of the phage-T5 procedure was necessary because T2-infected bacteria often lysed spontaneously on collection by centrifuging.

Phenol treatment of the lysate. This was performed by using a water-saturated solution of redistilled phenol. About 7ml. of 0·2M-Na_2HPO_4 was added to 100ml. of phenol solution so that the pH of the aqueous phase was 7·4. Usually an equal volume of cold phenol solution was added to the lysate (contained in a glass-stoppered tube) and the mixture was rocked back and forth by hand at a rate just sufficient to cause complete emulsification of the mixture in about 10min. The mixture was kept cold (in ice–water) and rocked for about 30min. before centrifuging (2°) at 2500*g* in a swing-out rotor for 20min. The upper aqueous phase was removed with a wide-bore Pasteur pipette, except when the DNA was required for physical measurements, when the phenol phase was removed and the solution containing DNA left in the tube. When there was an interface precipitate the aqueous layer was removed and the interface material obtained either by using a Pasteur pipette or by carefully removing the phenol layer so that the precipitate adhered to one side of the glass tube.

With lysates of T2r$^+$-infected bacteria 0·3vol. of aqueous phenol was used, and after centrifuging the emulsion at 2500*g* as above the phenol layer was removed and the

mixture of interface material and aqueous phase centrifuged (2°) at 5000*g* for 30 min. in a toughened glass tube. The clear supernatant was decanted from the precipitate.

The interface material was suspended evenly in 0·1 M-tris–HCl buffer, pH 8·0, containing EDTA (0·05 M). Residual phenol was removed from this suspension and from the aqueous phase by gently rocking with an equal volume of ice-cold ether with at least three changes. Residual ether was removed by rotary evaporation.

Removal of RNA from DNA preparations. RNA in the DNA preparations was degraded by incubation with ribonuclease (20 μg./ml.) at 37° for 30 min. The ribonuclease solution was previously heated at 80° for 20 min. to inactivate any possible deoxyribonuclease activity. The products of the ribonuclease digestion were often removed as completely as possible by dialysis against 0·5 M-NaCl in 0·05 M-phosphate (Na^+ and K^+ salts) buffer, pH 6·7.

Isolation of DNA from the interface precipitate. The interface suspension prepared as described above was incubated with lysozyme (300 μg./ml.) for 30 min. at 37°. Usually ribonuclease (10–20 μg./ml.) was included with the lysozyme for 15 min. A further incubation with 0·7% (w/v) sodium dodecyl sulphate for 5 min. at 37° usually improved the yield of DNA. The clear viscous solution was gently rocked with phenol for 30 min. as described, and the aqueous phase obtained by centrifuging was dialysed for 2 days with at least four changes against 1l. of 0·04 M-NaCl–0·002 M-EDTA, pH 8·0. The dialysed solution was centrifuged at 18000*g* for 20 min. to remove possible particulate material that had not been removed by the original centrifuging. Usually the faint precipitate contained only traces of DNA and radioactivity. Occasionally the procedure released a very small percentage of the interface DNA (10–20% instead of 40–90%). The reason for this failure of the procedure is unknown. The interface DNA preparation obtained by the above procedure contained only traces of protein but did contain fragments of RNA produced by the ribonuclease digestion. These contaminants did not interfere with the analytical procedures employed.

Centrifuging in a caesium chloride density gradient. This was performed in a Spinco model E analytical ultracentrifuge and in an MSE Speed 40 or a Spinco model L ultracentrifuge. Solutions for the analytical ultracentrifuge were prepared in 0·02 M-tris buffer, pH 8·0, and contained up to 4 μg. of each DNA/ml. and CsCl to make a density of 1·715 at 25°. The density of the solution was adjusted by using a refractometer and the cells were filled without special precaution to prevent shear degradation of the DNA. A 12 mm. standard cell and a 12 mm. cell with a negative wedge window were centrifuged together at 44770 rev./min. for 24 hr. at 25° in a rotor with a reference hole. An aperture mask was used so that photographs of each cell could be obtained, and the photographs were analysed in an Analytrol integrating densitometer (Beckman Instruments Inc., Palo Alto, Calif., U.S.A.). The buoyant density of each band was calculated (Sueoka, 1961) with reference to the position in the cell of the band of reference DNA (*M. lysodeikticus* DNA, $\rho = 1{\cdot}731$). Some experiments were performed at pH 11·0 with 0·05 M-phosphate buffer (Vinograd, Morris, Davidson & Dove, 1963).

For preparative density-gradient centrifuging the solutions contained DNA and CsCl to make a density of 1·70 ±0·01 at 2°. Density was measured by using a 200 μl. micropipette as a pycnometer. Three tubes were centrifuged at 110000*g* for 40 hr. at 2–4°. At the end of the run, fractions were collected from the tube by piercing the bottom with a needle and collecting fractions of 5 drops directly into polyethylene counting bottles.

Sedimentation of DNA in the Spinco model E ultracentrifuge. This was performed at 31410 rev./min. and 20·0°. The solutions contained about 20 μg. of DNA/ml. in 0·7 M-NaCl–0·05 M-phosphate buffer, pH 6·7 (Burgi & Hershey, 1961), and were very slowly introduced into a partly assembled 12 mm. standard cell by using a syringe with a wide-bore hole (no needle). Photographs were taken at 2 min. intervals and analysed by using a densitometer. All calculations were made by using the mid-point of the sedimenting boundary and the values obtained were corrected to that in water. On some occasions insufficient material was available to make a solution of concentration 20 μg. of DNA/ml. Because the sedimentation coefficient of DNA is dependent on concentration, the values obtained in these cases were corrected to the value at 20 μg./ml., by using as a basis the concentration-dependence of T5-phage DNA.

Zone centrifuging. This was performed by layering 0·5 μg. of labelled DNA on a 5–20% (w/v) linear gradient of sucrose in 2 M-NaCl and centrifuging in the SW 39 head of a Spinco model L ultracentrifuge for 3 hr. at 30000 rev./min. See Burgi & Hershey (1963) for details.

Chromatography of DNA. This was performed on columns of kieselguhr coated with methylated serum albumin as described by Mandell & Hershey (1960) and Lacks (1962). Linear gradients of increasing NaCl concentrations were obtained by using two reservoirs one of which acted as a mixing chamber. Usually about 1 mg. of DNA was applied to the column and 5 ml. fractions were collected. All operations were performed at room temperature, and air pressure of about 3 lb./in.^{2} was applied to increase the flow rate to about 30 ml./hr. Methylated serum albumin of decreased basicity was made as described by Mandell & Hershey (1960) except that methylation was stopped after 24 hr. instead of 72 hr. Only a slight precipitate had formed but good yields were obtained by precipitation of the remainder of the protein with ether.

Increase of extinction of DNA at 260 mμ on heating. This was measured in a Unicam SP.500 spectrophotometer fitted with a water-jacketed cell holder. Solutions for analysis contained 0·15 M-NaCl–0·015 M-sodium citrate and DNA (about 20 μg./ml.), and were first briefly evacuated to remove air bubbles.

Tritium content of samples of DNA. This was measured by mixing with the sample about 1 mg. of bovine plasma albumin, cooling the mixture in ice and adding cold $HClO_4$ to give a final concentration of 0·25 N. After a few minutes in ice the solution was centrifuged (2°) at 2500*g* for 15 min. and the precipitate washed once or twice with cold 0·2 N-$HClO_4$. The DNA was hydrolysed in 0·5 N-$HClO_4$ at 70° for 25 min. and the solution centrifuged. A portion (0·05 ml.) of the supernatant was added to 1·2 ml. of scintillation fluid and the samples were counted three times for 1000 sec. in a tritium coincidence scintillation counter (Isotope Developments Ltd., Beenham, Reading, Berks.). The standard error of the results was usually not more than 2%. The scintillation fluid contained 100 ml. of sulphur-free toluene, 100 ml. of dioxan, 60 ml. of methanol, 21 g. of naphthalene, 1·2 g. of 2,5-diphenyloxazole and 26 mg. of 1,4-bis-(5-phenyloxazol-2-yl)benzene. In some experiments

herring-sperm DNA was added as a co-precipitant instead of albumin, and fractions from the chromatographic columns containing NH_3 were first neutralized. From time to time internal standards were used to check possible quenching of the scintillators. The presence of $HClO_4$ in the mixture decreased the count rate of standards by about 50%, but apart from this no quenching was observed with samples of different origin. The efficiency of counting ^3H in the presence of the $HClO_4$ was about 10%.

Mechanical disruption of bacteria. This was performed with a bacterial press (Hughes, 1951) at −25°.

Chemical estimations. DNA was measured by the method of Burton (1956), with deoxyribose as standard. Only the purine-bound deoxyribose reacts in this method and so the values were doubled to obtain the amounts of DNA deoxyribose that are given in the paper. RNA was measured by the orcinol reaction under the conditions used by Ogur & Rosen (1950), with ribose as the standard. Again, the colour represents purine-bound ribose and so they were likewise doubled to give the results that are presented. Protein was measured by using the Folin–Ciocalteu phenol reaction (Lowry, Rosebrough, Farr & Randall, 1951), with bovine serum albumin (Armour Laboratories, Eastbourne, Sussex) as standard.

RESULTS

Analysis of the total DNA of pulse-labelled T5-infected E. coli

Cultures of T5-infected bacteria were pulse-labelled with [^3H]thymidine as described in the Materials and Methods section, and DNA was extracted in good yields (cf. Table 5) by incubating the cells with lysozyme, freezing and thawing three times, warming the lysate with 0·75% sodium dodecyl sulphate and subsequently deproteinizing the clear solution with water-saturated phenol. The solution was treated with ribonuclease and the properties of the DNA were investigated by using caesium chloride density gradients and chromatography on methylated albumin columns.

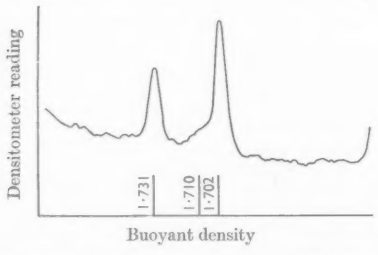

Fig. 1. Density-gradient centrifuging of DNA from T5-infected *E. coli*. DNA was isolated as described in the text from a culture 37 min. after infection, and 4 μg. of this DNA was centrifuged together with 1 μg. of *M. lysodeikticus* DNA ($\rho = 1{\cdot}731$) in CsCl solution (see the Materials and Methods section) in the ultracentrifuge. Photographs were analysed in a densitometer.

Density-gradient centrifuging. In a density gradient of caesium chloride in the analytical ultracentrifuge only one major component was detected in DNA obtained from T5-infected *E. coli* (Fig. 1). This component had a buoyant density of 1·702, which is identical with the reported value for DNA of purified T5 phage (Schildkraut, Marmur & Doty, 1962). It is evident that by 37 min. after infection most of the *E. coli* DNA ($\rho = 1{\cdot}710$) has been degraded, although significant amounts were found when the infected culture was harvested before 30 min. after infection.

Similar results to Fig. 1 were obtained in another experiment, performed in caesium chloride buffered at pH11.0 with 0·05M-phosphate buffer. Under these conditions single-stranded DNA has a considerably increased buoyant density (Vinograd *et al.* 1963).

Chromatography of pulse-labelled DNA from T5-infected E. coli. Chromatography of DNA from pulse-labelled T5-infected *E. coli* on columns of kieselguhr coated with methylated serum albumin resolved the DNA preparation into components of differing specific activity (counts/sec./$E_{260m\mu}$) (Fig. 2). About 50% of the applied DNA was eluted as a heterogeneous peak with 0·7M-sodium chloride (peak 1) and a second smaller peak (peak 2) was obtained by increasing the salt concentration and pH of the eluting fluid. The remainder of the DNA

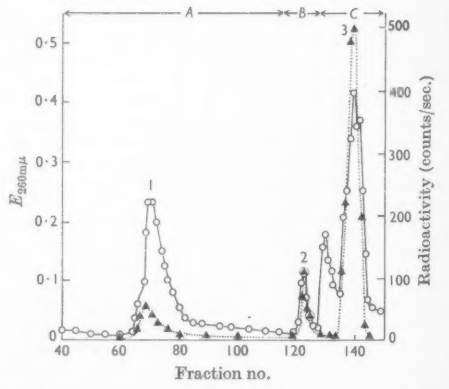

Fig. 2. Chromatography of DNA from pulse-labelled T5-infected *E. coli*. DNA was isolated as described in the text from a culture pulse-labelled at 33 min. and subsequently treated with ribonuclease. Then 1·1 mg. of this DNA in 50 ml. of 0·65M-NaCl in 0·05M-phosphate buffer, pH 6·7, was adsorbed on to a 20 cm. × 2 cm. diam. column and eluted with: (*A*) a linear gradient of 0·65–0·8M-NaCl in 0·05M-phosphate buffer, pH 6·7; (*B*) 1·5M-NaCl in 0·1M-tris buffer, pH 8·0; (*C*) 1·5M-NaCl in 2N-NH_3. Fractions of volume 5 ml. were collected. ○, $E_{260m\mu}$; ▲, radioactivity.

could be eluted only with 2N-ammonia–1·5M-sodium chloride (peak 3). Occasionally a fourth peak was obtained when changing from the buffered salt to the ammoniacal solution, but this was not usually observed and its significance is unknown. The ^3H content of the peaks differed markedly; peaks 2 and 3 had a similar specific activity and contained about 80% of the total ^3H. Peak 1 was heterogeneous; the leading edge consistently contained DNA of higher specific activity than the remainder of the peak, the specific activity of which was less than 10% that of peaks 2 and 3. Recovery of DNA from the column measured as 260mμ-absorbing material usually accounted for up to 90% of the applied material, and only traces of RNA were observed in the peaks.

The fractions comprising the peaks were pooled, neutralized where necessary, concentrated and dialysed. Measurements of radioactivity and deoxyribose content (Table 1) substantiated the differences observed in Fig. 2. Since the DNA of peak 1 was eluted at the salt concentration that would be required to elute T5-phage DNA, the sedimentation coefficients of the DNA of selected fractions were determined and are listed in Table 2. The DNA from the leading edge of peak 1 had a relatively low sedimentation coefficient, whereas the remainder of peak 1 had a sedimentation coefficient similar to that of DNA from T5 phage. The shape of the sedimenting boundaries of the DNA from the leading edge of peak 1 and from peak 2 suggested that these fractions were much more polydisperse than the DNA of the centre fractions of peak 1. The boundary of this material was of similar shape to that obtained with native T5-phage DNA.

The labelling pattern observed in Fig. 2 suggested that peak 3 may contain DNA that is a precursor of the DNA in peak 1. To test this idea a culture of T5-infected *E. coli* was pulse-labelled with [^3H]-thymidine 27min. after infection, by stopping the incorporation of ^3H after 30sec. with a large excess of unlabelled thymidine. The culture was aerated for a further 10min., the bacteria were collected and the DNA was isolated from a lysozyme lysate. A sample of this DNA preparation was incubated

Table 1. *Specific activity of fractions from the column*

Fractions from the column described in Fig. 2 were combined as indicated, neutralized, concentrated by rotary evaporation and dialysed against 0·7M-NaCl in 0·05M-phosphate buffer, pH 6·7, and the DNA was precipitated in 0·2N-$HClO_4$. Deoxyribose and radioactivity were measured in each fraction.

Fraction no.	Total deoxyribose (μmole)	Total radioactivity (counts/sec.)	Specific activity (counts/sec./μmole of deoxyribose)
60–68	0·053	46·5	886
70–73	0·551	147·5	273
74–80	0·365	95·0	260
122–125	0·120	225·0	1875
129–134	0·114	31·5	276
136–143	0·900	2710·0	3010

Table 2. *Sedimentation coefficients of DNA samples from the column*

Fractions from the column were obtained as described in Table 1 and diluted when necessary in 0·7M-NaCl in 0·05M-phosphate buffer, pH 6·7. Sedimentation coefficients were obtained in the analytical ultracentrifuge and corrected to $S_{20,w}$ at 20μg. of DNA/ml., by using as a basis the concentration-dependence of the sedimentation coefficient of T5-phage DNA.

Fraction no.	Concn. of DNA (μg./ml.)	$S_{20,w}$ (observed) (s)	$S_{20,w}$ (corrected) (s)
60–68	11·5	33	31
70–73	21·0	45	45
74–80	18·0	45	44·5
122–125	13·5	42	40
T5-phage DNA	20·0	44	44

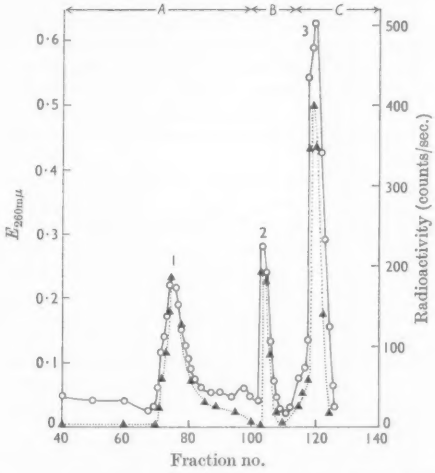

Fig. 3. Chromatography of DNA from a culture of T5-infected *E. coli* pulse-labelled 10min. before collection of the bacteria. The culture was pulse-labelled as described in the text and the DNA isolated. Then 1·1mg. of the DNA was adsorbed on to a column and eluted as described in Fig. 2 except that buffered 0·55M-NaCl was used for adsorption and a linear gradient of 0·55–0·77M-NaCl in buffer was used. ○, $E_{260m\mu}$; ▲, radioactivity.

with ribonuclease and applied to a chromatographic column and eluted as before (Fig. 3). The DNA of peak 3 was of slightly lower specific activity than that of peak 1, which is consistent with the idea that the former is a precursor of the latter. We were not able to recover the DNA of peak 3 from the column without the use of 2N-ammonia. With the described columns the use of saturated sodium chloride, saturated ammonium sulphate and 1·5M-sodium chloride buffered at pH 9·6 was unsuccessful, as was an attempt with a column with methylated serum albumin that was less extensively methylated than that described by Mandell & Hershey (1960).

The chromatographic results described suggested that the pulse-labelled DNA was of an unusual structure, but no unusual properties of this DNA were detected. All the DNA samples of fractions from the column denatured on heating at the same temperature, and the magnitude of the hyperchromic effect on heating was similar in all cases except that for the DNA of peak 3, which showed a rather smaller increase (25% instead of 30–33% for the other samples), but these fractions were contaminated with protein from the column. No differences in buoyant density in caesium chloride were detected; all the DNA samples had a buoyant density of 1·702 even when centrifuged at pH11·0.

Since it is known that DNA damaged by various procedures can be firmly bound to these chromatographic columns (Hershey, Goldberg, Burgi & Ingraham, 1963), it was important to establish whether or not the observed retention of the highly labelled DNA was an artifact of the procedures employed for the characterization and isolation of replicating DNA. To test the idea that the retention was a result of damage caused by the decay of ^3H in the DNA, a sample of DNA was prepared from purified T5 phage labelled with [^3H]thymidine. The resultant DNA preparation had a specific activity of 1130 counts/sec./μmole of deoxyribose. It was subjected to chromatography 3 days after preparation and again 2 months later. In both cases about 90% of the applied DNA could be eluted with sodium chloride solutions, pH 6·7, alone (Table 3). This recovery is similar to that obtained with unlabelled phage DNA and therefore ^3H decay cannot be responsible for the aforementioned chromatographic effects.

Another possible explanation for the retention is that replicating DNA is susceptible to nuclease action during preparation of the lysates, whereas DNA contained inside phage heads would be refractory. The following experiments were designed to examine this possibility. A sample of ^3H-labelled T5-phage DNA was added to a suspension of unlabelled T5-infected *E. coli* and the bacteria were lysed with sodium dodecyl sulphate (see the Materials and Methods section). The lysate was treated with water-saturated phenol as described and the aqueous-phase fraction was collected. The solution was dialysed to remove phenol and then digested with ribonuclease. After further dialysis against 0·5M-sodium chloride a sample of the DNA was subjected to chromatography and the amount of labelled DNA that could be eluted only with 2N-ammonia was measured. This proportion (sample 3, Table 3) was similar to that observed with labelled T5-phage DNA alone. A similar experiment was performed by adding labelled T5-phage DNA to a lysozyme lysate of T5-infected bacteria and incubating at 37° for 5min. before adding sodium dodecyl sulphate (0·75%) and isolating the DNA in the usual manner. In this case rather more labelled DNA was firmly bound to the column (sample 5, Table 3). However, we do not consider that this behaviour was due to nuclease action on the labelled DNA during the incubation with the lysozyme lysate, because of the behaviour of sample 4 (Table 3). In this case, the labelled T5-phage DNA was mixed with unlabelled interface DNA that was obtained from a sodium dodecyl sulphate lysate as described in the next section. The interface DNA is firmly bound to the column and at the same time increases the proportion of labelled T5-phage DNA that is also bound in this way.

Table 3. *Retention of DNA on chromatographic columns*

Samples (1mg.) of each of the following DNA preparations (see the text for details) were applied to chromatographic columns and the DNA was eluted as described in Fig. 2. The percentage of the labelled DNA that could be eluted only with 2N-NH_3 was measured.

DNA sample applied	Labelled DNA retained (% of total)
1. Tritium-labelled T5-phage DNA 3 days after preparation	11·5
2. Tritium-labelled T5-phage DNA 2 months after preparation	11·5
3. Tritium-labelled T5-phage DNA added to a suspension of T5-infected *E. coli* before lysing with sodium dodecyl sulphate	11·7
4. Tritium-labelled T5-phage DNA (500μg.) and unlabelled interface DNA (400μg.)	20·6
5. Tritium-labelled T5-phage DNA added to a lysozyme lysate of T5-infected *E. coli* and incubated at 37° for 5min.	24

Fractionation of DNA from phage-infected E. coli *by phenol extraction of sodium dodecyl sulphate lysates*

When a suspension of T5-infected *E. coli* was lysed at 37° with 0·75% sodium dodecyl sulphate and the viscous lysate rocked with water-saturated

phenol and centrifuged, a copious white precipitate appeared at the interface between the aqueous and phenol layers. This interface precipitate contained an appreciable amount of DNA. When T5-infected bacteria were pulse-labelled with [^{3}H]thymidine for 30sec. at 35min. after infection, the interface material contained about half the DNA and as much as 90% of the radioactivity that was incorporated into DNA (Table 4).

This fractionation of the phage DNA was very reproducible and was unaffected by the rate and time of rocking of the lysate with phenol. The amount of sodium dodecyl sulphate used for lysis did not affect the subsequent fractionation, although some DNA was extracted from bacteria that had not been lysed with detergent. If the infected bacteria were first lysed with lysozyme before treatment with sodium dodecyl sulphate and phenol, very little interface material was found; almost all the DNA was found in soluble form in the aqueous layer, and the specific activity of this DNA in the interface fraction was close to that in the aqueous layer (Table 5). Similarly, the fractionation was not obtained if the infected bacteria were broken in a bacterial press (Table 5).

Conversion of interface DNA into aqueous-phase DNA during multiplication of phage. To test the hypothesis that the interface DNA was a precursor of the aqueous-phase DNA, a culture of T5-infected *E. coli* was supplied with [^{3}H]thymidine 29min. after infection, and a large excess of unlabelled thymidine was added 30sec. later. At intervals thereafter samples of the culture were removed, the bacteria were collected and lysed with sodium dodecyl sulphate, and the lysates were rocked with phenol. The changes in ^{3}H and DNA content of the aqueous-phase and interface fractions obtained are shown by Fig. 4.

For the period between 29 and 40min. after infection the interface DNA content remained constant, whereas the DNA content of the aqueous-phase fraction increased linearly. Initially, most of the radioactivity of the pulse was found in the interface fraction, but this was subsequently transferred to the aqueous-phase fraction. Approx. 50% of the peak radioactivity was transferred in 6min.

A similar experiment, pulse-labelled at 18min. after infection, showed essentially the same pattern of ^{3}H transfer.

Changes in DNA content of the aqueous and interface pools of DNA after infection with T5 phage.

Table 4. *Fractionation of the DNA from a sodium dodecyl sulphate lysate of pulse-labelled T5-infected* E. coli

A sodium dodecyl sulphate lysate (see the text for details) of pulse-labelled infected *E. coli* was rocked with an equal volume of water-saturated phenol (see the Materials and Methods section) for 1hr. The emulsion was centrifuged and the aqueous phase and the interface precipitate were separated. The precipitate was suspended in 0·1M-NaCl and the acid-insoluble deoxyribose and radioactivity were measured in both fractions after removal of phenol by ether extraction.

Fraction	Total deoxyribose (μmoles)	Total radioactivity (counts/sec.)	Specific activity (counts/sec./μmole of deoxyribose)
Aqueous phase	1·20	1560	1300
Interface	0·92	12200	13250

Table 5. *Effect of prior lysozyme treatment and mechanical breakage of T5-infected cells on the phenol fractionation*

Expt. *A*. A suspension of T5-infected *E. coli* (pulse-labelled at 33min.) was divided into two samples. One was lysed with sodium dodecyl sulphate as described in Table 4; the other was incubated at 37° with lysozyme (300μg./ml.) and frozen and thawed three times before treatment with sodium dodecyl sulphate. Expt. *B*. Two unequal portions of a similar suspension were obtained and treated as in Expt. *A*, except that instead of lysing one portion with lysozyme the cells were broken in a bacterial press at −25°. In both experiments the lysates were fractionated with phenol and assayed as described in Table 4.

Method of disruption	Fraction	Total radioactivity (counts/sec.)	Total deoxyribose (μmoles)	Specific activity (counts/sec./μmole of deoxyribose)
A. Sodium dodecyl sulphate alone	Aqueous	310	1·06	290
	Interface	1140	0·51	2240
Lysozyme and sodium dodecyl sulphate	Aqueous	1230	1·50	820
	Interface	50	0·06	835
B. Sodium dodecyl sulphate	Aqueous	113	0·76	148
	Interface	607	0·92	660
Mechanical disruption	Aqueous	1350	2·84	476
	Interface	18	<0·03	—

Since the preceding results indicated that the interface DNA fraction contained the replicating DNA, it was decided to measure the amount of DNA in the interface and aqueous-phase fractions at various times after infection of the bacteria with T5 phage. Samples of a culture of T5-infected *E. coli* were removed at intervals from 18min. after infection, and the distribution of DNA was measured as before. The results of this experiment are shown by Fig. 5. The DNA content of the interface fraction increased in the period between 18 and 30min. after infection, reaching a constant value of about 55 T5-phage DNA molecules/bacterium. DNA began to accumulate in the aqueous phase from about 23min. after infection, and by 30min. the increase in size of this pool accounted for most of the DNA synthesis of the culture. At 18 and 23 min. after infection 70% and 40% respectively of the aqueous-phase DNA was *E. coli* DNA as determined by caesium chloride density-gradient centrifuging.

Before 15min. after infection the aqueous-phase fraction and the interface fraction had a similar DNA content, most of which would be *E. coli* DNA.

Fractionation of the DNA by phenol is probably due to condensation of the DNA. Published studies by electron microscopy of developing phage T2 (Kellenberger, 1961) prompted the idea that the

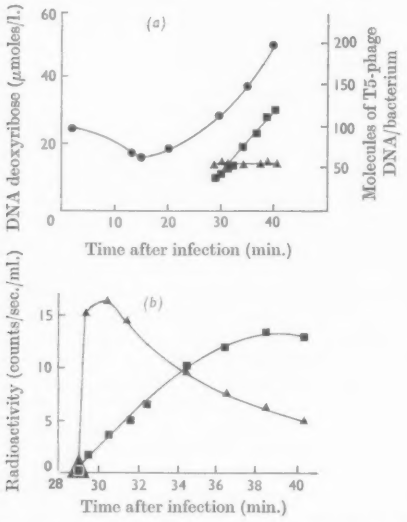

Fig. 4. Changes in (*a*) DNA and (*b*) ^3H content of the aqueous-phase and interface fractions after a pulse of [^3H]-thymidine. Experimental details are described in the text. ●, Total DNA; ▲, interface DNA; ■, aqueous-phase DNA.

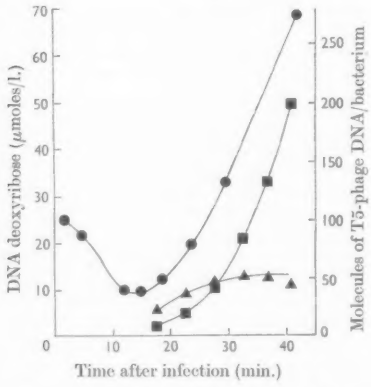

Fig. 5. Changes in the distribution of DNA between the aqueous-phase and interface fractions after infection with T5 phage. Experimental details are given in the text. ●, Total DNA; ▲, interface DNA; ■, aqueous-phase DNA.

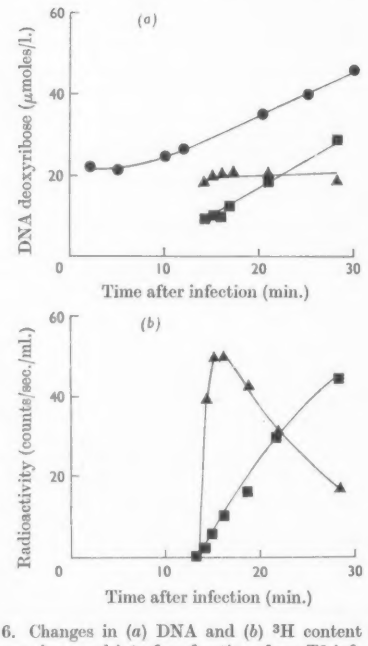

Fig. 6. Changes in (*a*) DNA and (*b*) ^3H content of the aqueous-phase and interface fractions from T2-infected *E. coli* after a pulse of [^3H]thymidine. Experimental details were similar to those described for Fig. 4. ●, Total DNA; ▲, interface DNA; ■, aqueous-phase DNA.

DNA in the aqueous-phase pool might be derived from that phage DNA which had condensed to form phage heads. If this were so, inhibition of DNA condensation should alter the normal distribution of DNA between the aqueous phase and interface of phenol-treated lysates of infected bacteria.

Since chloramphenicol inhibits DNA synthesis in T5-infected bacteria (Pfefferkorn & Amos, 1958; Crawford, 1959), T2-infected cultures were used for the following experiments. Chloramphenicol is known to inhibit the condensation process in this system (Kellenberger, Séchaud & Ryter, 1959).

In the absence of chloramphenicol (Fig. 6) the distribution of DNA and radioactivity between the aqueous and interface fractions was very similar to that obtained with the T5-phage system described above. The DNA content of the interface fraction remained constant, whereas that of the aqueous-phase fraction increased at the rate of overall DNA synthesis. Further, ^3H initially entered the interface fraction and was subsequently transferred to the aqueous-phase fraction. When chloramphenicol (50μg./ml.) was added 11min. after infection, a completely different situation was found (Fig. 7). The increase in total DNA could be completely accounted for by the increase in DNA content of the interface fraction, whereas that of the aqueous phase remained constant. Also, most of the radioactivity incorporated from the pulse of [^3H]thymidine entered the interface and stayed there throughout the experiment. This finding is consistent with the idea that the interface fraction of DNA consists of the fibrillar DNA that has not been condensed to form phage heads.

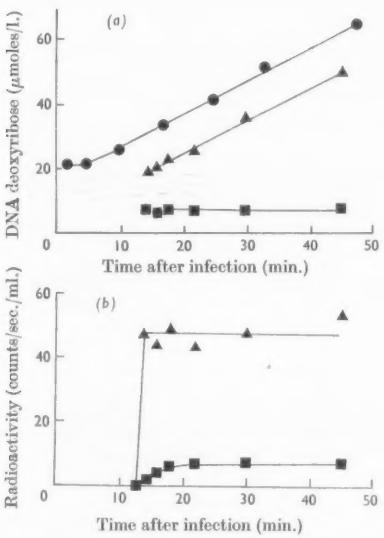

Fig. 7. Effect of chloramphenicol on the distribution of (*a*) DNA and (*b*) ^3H between the interface and aqueous-phase fractions of T2-infected *E. coli*. Experimental details were similar to those described for Fig. 6 except that chloramphenicol (final concn. 50μg./ml.) was added 11min. after infection. ●, Total DNA; ▲, interface DNA; ■, aqueous-phase DNA.

Release of DNA from the interface fraction

The interface fraction obtained from T5-infected bacteria by phenol fractionation contained appreciable amounts of RNA and protein as well as DNA (e.g. 1·5μmoles of RNA ribose and 4·2mg. of protein/μmole of DNA deoxyribose). The DNA was not extracted from interface suspensions by various combinations of heating at 55°, 2M-sodium chloride and pH10·6. The suspension was partly clarified by treatment with papain but the DNA was still associated with the insoluble material. On centrifuging in a caesium chloride density gradient, virtually all of the radioactivity was associated with the insoluble material on the surface. After fixation with osmic acid, examination in an electron microscope showed that the interface fraction contained many particles of similar shape and size to bacteria. This suggested that the DNA was not extracted by the above procedures because it was contained by the structures of the bacterial cell wall, which are resistant to the detergent and phenol.

The interface precipitate was found to be susceptible to lysozyme and this enzyme released 40–75% of the DNA in a soluble form. The specific activity of the released DNA was similar to that of the residual DNA that was not released from the interface material (Table 6). As much as 90% of the DNA has been released in some of the experiments in which sodium dodecyl sulphate was added to 0·3% (w/v) concentration after the lysozyme incubation and incubation continued at 37° for 5min.

A sample of interface DNA that was released by detergent as described above was treated with ribonuclease and chromatographed on a column of kieselguhr coated with methylated serum albumin. This DNA, like part of the DNA from a lysozyme lysate of T5-infected bacteria, was firmly bound by the chromatographic column and could be eluted only with ammonia (Fig. 8). By contrast, DNA from the aqueous-phase fraction could be eluted with sodium chloride solutions at pH6·7.

The ^3H-labelled DNA isolated from the interface fraction was mixed with DNA isolated from ^{32}P-labelled T5 phage and centrifuged in a density gradient of caesium chloride. Fig. 9 shows that the distributions of ^{32}P and ^3H coincide and that there

Table 6. *Release of DNA from the interface precipitate by lysozyme*

Interface material was prepared from pulse-labelled T5-infected *E. coli* as described in Table 4, and evenly suspended in 0·1 M-tris buffer, pH 8·0, containing EDTA (0·06 M). Residual phenol was removed by three ether extractions and final traces of ether were removed by evacuation. Samples of this suspension were incubated at 37° for 30 min. in the presence and absence of lysozyme (final concn. 300 μg./ml.) and then subjected to a further phenol extraction. The ^3H and deoxyribose contents of the resultant fractions were measured.

Treatment	Fraction	Total deoxyribose (μmole)	Total radioactivity (counts/sec.)	Specific activity (counts/sec./ μmole of deoxyribose)
Control	Aqueous	0·068	72	1060
	Interface	0·314	1030	3280
Lysozyme	Aqueous	0·319	956	3000
	Interface	0·104	308	2960

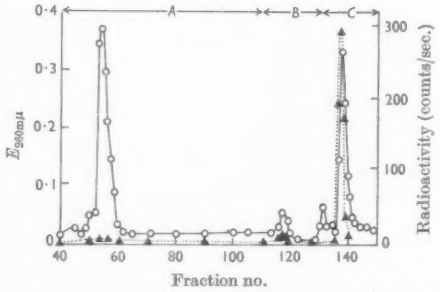

Fig. 8. Chromatography of interface DNA and DNA from T5 phage. DNA was isolated from labelled interface as described in Table 6. Then 300 μg. of this DNA and 400 μg. of unlabelled DNA from T5 phage were applied to a chromatographic column (as described in Fig. 2) with (*A*) a linear gradient of 0·65–0·95 M-NaCl in 0·05 M-phosphate buffer, pH 6·7; (*B*) 2 M-NaCl in 0·1 M-tris buffer, pH 8·0; (*C*) 2 M-NaCl in 2 N-NH_3. ○, $E_{260m\mu}$; ▲, radioactivity.

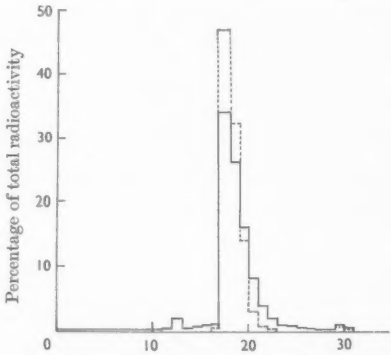

Fig. 9. Density-gradient centrifuging of ^3H-labelled interface DNA and ^{32}P-labelled T5-phage DNA. The CsCl solution (1·5 ml.) contained 0·15 μg. of T5-phage DNA and 2·6 μg. of interface DNA and was centrifuged for 46 hr. (see the Materials and Methods section). Fractions were collected directly into counting vials and the ^3H and ^{32}P contents were measured. ——, ^3H; – – – –, ^{32}P. The graph shows, for each isotope, the percentage of the total radioactivity that was found in each fraction.

is no tritium at the top or the bottom of the density gradient. This result shows that the DNA released from the interface fraction is not attached to measurable amounts of protein.

To obtain an indication of the size of the interface DNA, a ^3H-labelled sample was centrifuged together with a sample of ^{32}P-labelled T5-phage DNA in a 5–20% (w/v) gradient of sucrose in 2·0 M-sodium chloride. Fig. 10 shows that approx. 50% of the ^3H-labelled DNA sedimented as a broad band much faster than the sharp band of the ^{32}P-labelled marker DNA. This sedimentation pattern was unaffected by the sodium chloride concentration in the sucrose and the amount of DNA applied (in the range 0·2–1·5 μg.) and cannot be due to non-specific aggregation since the T5-phage DNA sedimented normally. The sedimentation behaviour is not due to any association with a large bulk of protein or other non-DNA material because essentially the same sedimentation results were obtained with DNA that had been recovered from an equilibrium centrifugation in caesium chloride.

DISCUSSION

Although the chromatographic experiments and the phenol fractionation indicated that an appreciable amount of the total DNA of T5-infected *E. coli* is replicating, caesium chloride density-gradient centrifuging with the total DNA of lysozyme lysates and with the purified interface DNA revealed

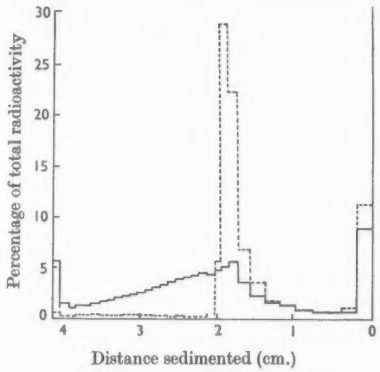

Fig. 10. Zone sedimentation of ^{3}H-labelled interface DNA and ^{32}P-labelled T5-phage DNA. A mixture of 0·4 μg. of ^{3}H-labelled interface DNA and 0·1 μg. of ^{32}P-labelled T5-phage DNA was layered on a 5–20% (w/v) sucrose gradient and centrifuged as described in the Materials and Methods section. Fractions were collected by piercing the tube and collecting drops directly into counting vials. The graph shows, for each isotope, the percentage of the total radioactivity that was found in each fraction. ——, ^{3}H; – – – –, ^{32}P.

no components having an unusual buoyant density. The same conclusion was reached with experiments performed at pH11·0, at which pH single-stranded DNA has a much greater density than at neutral pH (Vinograd *et al.* 1963).

Pulse-labelled DNA of T5-infected bacteria therefore differs from that of non-infected *E. coli*, where components of unusual buoyant density have been detected (Rolfe, 1963; Rosenberg & Cavalieri, 1964). In these cases the unusual components were detected only after special treatments and it is conceivable that the techniques employed in the present study were inadequate. In any case, if single-stranded regions are present in molecules of replicating T5-phage DNA these regions must be very short compared with the length of the molecule. This conclusion is in accord with present evidence on DNA replication in *E. coli*, which suggests that only a minute portion of the chromosome is involved with replication at any specified time (Cairns, 1963).

Chromatography of the total DNA of phage-infected bacteria on columns of kieselguhr coated with methylated serum albumin resolved the DNA into two major components of different specific activity. The leading edge of peak 1 (Fig. 2) apparently contained fragments of DNA molecules, whereas the DNA of the remainder of the peak was probably derived from intracellular phage particles. The highly labelled DNA, on the other hand, was firmly bound to the column and could be eluted in good yield only by 2N-ammonia. Hershey *et al.* (1963) have shown that DNA will bind to these columns in a similar manner if it is damaged by any of several methods, which include decay of incorporated radioactive atoms, intense u.v. light and localized denaturation in internal regions of the molecule caused by shear forces. In the present study ^{3}H decay cannot be responsible for the retention because a sample of ^{3}H-labelled T5-phage DNA was eluted normally and in the pulse–chase experiment the DNA of peak 1 had a rather higher specific activity than the DNA of peak 3.

Similarly, no evidence has been obtained to suggest that specific nuclease action on replicating DNA during lysis of the bacteria was responsible for the effect, although the experiments could not negate the possibility of nuclease action exclusively at the moment of lysis of the bacterial cell. It is more likely that the retention of the highly labelled DNA by the column is a consequence of the physical configuration of replicating DNA. Conceivably, either forked molecules or short single-stranded regions in the molecule where base-pairing of incoming nucleotides was about to occur could be responsible.

The fractionation of the DNA of phage-infected bacteria with phenol was reproducible and provided a convenient method for the selective isolation of pulse-labelled DNA. That this DNA fraction contained replicating DNA was demonstrated by the kinetics of ^{3}H transfer from the interface fraction to the aqueous-phase fraction. The kinetics differ somewhat from the ideal case (Zilversmit, Entenman & Fishler, 1943) in that the specific activity of the interface fraction decreased rather more slowly with time. This divergence is not unexpected in a complex system involving the synthesis of phage DNA and the assembly of phage particles, since each DNA molecule will need to remain in the replicating pool for an appreciable time and, further, the specific activity of the aqueous-phase DNA is not necessarily a measure of the specific activity of the immediate product of replication. Moreover, some of the completed DNA must remain in the replicating pool to act as templates for the synthesis of new material.

The ability to separate a fraction of DNA from phage-infected bacteria that is enriched in replicating DNA allowed some measure to be made of the pool size of replicating DNA at different times after infection. The results obtained with T5-infected bacteria are similar to those obtained with T2-infected bacteria by Hershey (1953) in that the pool of replicating (or precursor) DNA first increases in size and eventually reaches a constant value. In T5-infected bacteria this value was 55 T5-phage DNA molecules/bacterium (assuming a T5-phage

particle contains $1{\cdot}8 \times 10^{-16}$g. of DNA; Sinsheimer, 1960), and in the T2-phage system it was 60 T2-phage DNA molecules/bacterium. The latter value is higher than previous estimates of 17 in a synthetic medium and 35 in nutrient broth (Koch & Hershey, 1959). However, comparison is difficult because of the different growth conditions employed, and also it is possible that the interface fraction contained bacterial DNA and/or phage DNA that was not involved in the process of replication.

If T5-phage DNA replicates with only one growing point/T5-phage DNA molecule, and if it is assumed that two-thirds of the interface DNA acts as a template for the synthesis of new DNA (since the average template strand will be associated with half a strand of new material), the constant size of the interface pool of 55 T5-phage DNA molecules/bacterium and the constant rate of increase of the aqueous-phase pool at 10 T5-phage DNA molecules/bacterium/min. provide an estimate of 3·7min. for the maximum time of synthesis of a T5-phage DNA molecule under the described growth conditions. Similar considerations with the less reliable values from the T2-phage system give an estimate of 10min., which is close to a previous estimate of 9min. (Koch & Hershey, 1959).

The accumulation of DNA in the aqueous-phase fraction with time, and the observation that ^{3}H from a short pulse of [^{3}H]thymidine is transferred from the interface fraction, suggest that the DNA in the aqueous phase may be derived from completed phage particles. This conclusion was supported by the observation that in T2-infected cells chloramphenicol, which is known to inhibit the condensation of fibrillar replicating DNA to form phage heads (Kellenberger *et al.* 1959), also inhibited the production of aqueous-phase DNA. However, chloramphenicol may affect processes other than condensation, since unusually large DNA molecules have been found in chloramphenicol-treated T2-infected *E. coli* (Frankel, 1962). It is not known whether or not the DNA in the aqueous-phase fraction is derived solely from infective intracellular phage. It is perhaps reasonable to suppose that the phenol procedure cannot differentiate between mature infective particles and any immature particles in which the head portion has been completely assembled.

The phenol fractionation was not observed if the infected bacteria were lysed with lysozyme before treatment with sodium dodecyl sulphate and phenol, or if the cells were broken in a bacterial press (Table 5). These facts suggest that the integrity of some part of the bacterial cell wall is an important factor in the separation. Bolle & Kellenberger (1958) showed that *E. coli* lysed with sodium lauryl sulphate retain the characteristic rod-shaped morphology. It is also known that the rigid layer of *E. coli* cell walls is unaffected by detergent or phenol treatment but is degraded by lysozyme (Weidel, Frank & Martin, 1960). In the present study the interface precipitate contained bacteria-like structures; and of several procedures tried (including digestion with papain) incubation with lysozyme was the only method that released the DNA in a soluble form.

It appears possible that the replicating DNA may be trapped within the rigid-layer framework of the bacterial cell wall. DNA that has been condensed into phage-head structures may conceivably pass through smaller holes than the replicating DNA, which has both a higher molecular weight and a more extended fibrillar structure. Replicating bacterial DNA may be attached to the bacterial cell wall (Jacob, Brenner & Cuzin, 1963) and it is conceivable that the replicating phage DNA may be attached in a similar manner. As yet, our experiments give no clear indication about this possibility.

The DNA isolated from the interface fraction, like part of the DNA from a lysozyme–sodium dodecyl sulphate lysate of T5-infected cells, was completely retained by the chromatographic column. Although the cause of this retention is not known, the experiment showed that the interface fraction is free of normal T5-phage DNA. By contrast, interface DNA had the same buoyant density in caesium chloride as T5-phage DNA. This fact means that within the limits of the experiment the interface DNA was not covalently linked to protein.

Sedimentation studies in sucrose density gradients revealed that only a small amount of the isolated DNA was smaller than normal T5-phage DNA, suggesting that extensive degradation of the DNA had not occurred during isolation. The most striking observation from the sedimentation studies was that more than 50% of the DNA sedimented as a broad band appreciably faster than T5-phage DNA. The sedimentation behaviour is not due to any association with a large bulk of protein or other non-DNA material. If the relationship between sedimentation rate and molecular weight derived by Burgi & Hershey (1963) is valid for this material, the molecular weight of the fastest-sedimenting components (3·3cm. from the top) is about five times that of T5-phage DNA. It is also possible that the rapid sedimentation is a result of a change in configuration of the DNA associated with replication or recombination or both. This large DNA is reminiscent of that obtained by Frankel (1962) from T2-infected cells treated with chloramphenicol, but differs in that the T5-phage interface DNA is firmly bound by the chromatographic column. The question remains whether or not such DNA is itself part of an even larger structure that was degraded during isolation.

We thank Professor Sir Hans Krebs, F.R.S., for his encouragement and interest. We are also grateful to Miss A. West for making electron-microscope observations of the interface material. M. G. S. thanks The Royal Commission for the Exhibition of 1851 and the Damon Runyon Memorial Fund for an Overseas Science Research Scholarship and a Cancer Research Fellowship respectively, and also Dr A. D. Hershey and Dr E. Burgi for hospitality, advice and helpful discussion. The work at Oxford was aided by grants from The Rockefeller Foundation and the U.S. Public Health Service.

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Cholesterol in Human Semen

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(*Received* 10 *May* 1965)

1. The cholesterol content of human seminal plasma has been estimated in 200 samples. The mean concentration is 46·8mg./100ml. (S.E.M. ± 1·1, range 15–88). The free cholesterol corresponds to about 40% of the total. 2. Cholesterol in human semen is secreted almost entirely from the prostate gland.

Studying the biochemistry of human semen, Goldblatt (1935) demonstrated a cholesterol content of about 80mg./100ml. Scott (1945), who came to almost the same result, speculated about the origin of cholesterol in semen and proposed that two-thirds came from the seminal vesicles and one-third from the prostate gland. These proportions were calculated from analyses of the cholesterol content in prostate gland, prostatic fluid and seminal plasma.

During recent years the methods for determination of cholesterol have improved significantly. It has also been demonstrated that erroneous results with regard to the source of various compounds in the seminal plasma may be obtained if calculations are based on tissue extracts (Eliasson, 1959, 1964). In the present study the occurrence and origin of cholesterol in human seminal plasma have been studied.

MATERIAL AND METHODS

Semen samples were obtained from volunteers and the Sterility Laboratory of the Karolinska Hospital. Part of each sample was transferred to glass tubes and kept at −20° until analysed, usually within 2 weeks. Before analysis the samples were thawed at room temperature and centrifuged to remove the spermatozoa.

The total cholesterol was determined by the method of van Boetzelar & Zondag (1960). A 5ml. volume of reagents [made by mixing 4·0g. of toluene-*p*-sulphonic acid, 60ml. of acetic anhydride and 40ml. of acetic acid (99–100%), all reagent grade] was transferred to a 50ml. test tube and 1ml. of conc. H_2SO_4 (95–97%, reagent grade) was carefully added. The mixture was cooled to room temperature and 0·2ml. of seminal plasma added. The green colour was measured within 5–30min. at 620mμ against water in a Beckman DB or Beckman B spectrophotometer. Free cholesterol was precipitated by digitonin as described by Sperry & Webb (1950) and determined by the method of van Boetzelar & Zondag (1960). Recovery was studied after the addition of known amounts of cholesterol in acetic anhydride to the seminal plasma. The origin of cholesterol was studied by the 'split-ejaculation method' (for details see Eliasson 1959, 1964).

RESULTS

The accuracy of the method for measuring cholesterol was determined from ten analyses of a pooled semen sample. The standard error of the mean was ±0·3mg./100ml. The mean concentration of cholesterol in 200 samples was 46·8mg./100ml. (S.E.M. ± 1·1, range 15–88mg./100ml.). The recovery of added amounts of cholesterol was 92–94%. Free and total cholesterol were determined in ten randomly selected samples: 41% (S.E.M. ± 1·5) of the cholesterol occurred in the free form.

The origin of cholesterol was studied in 'split-ejaculates' from three volunteers. The results demonstrated that probably all the cholesterol was secreted from the same origin as acid phosphatase, i.e. the prostate gland. The experimental results

Table 1. *Concentration of various constituents in different portions of human seminal fluid*

Fraction no.	Weight (g.)	Acid phosphatase (units/ml.)	Cholesterol (mg./100ml.)	Fructose (mg./100ml.)
A1	0·17	11·330	114	72
2	0·46	3·840	76	205
3	0·35	5·375	80	120
4	0·37	3·650	50	300
5	0·54	2·750	43	312
6	1·35	1·215	30	432
B1	0·82	6·655	96	120
2	0·60	5·120	54	230
3	0·40	3·010	46	250
4	0·44	2·240	40	290
5	0·68	2·495	36	310
C1	1·02	7·680	90	110
2	1·29	1·750	35	495
3	1·43	1·560	25	565

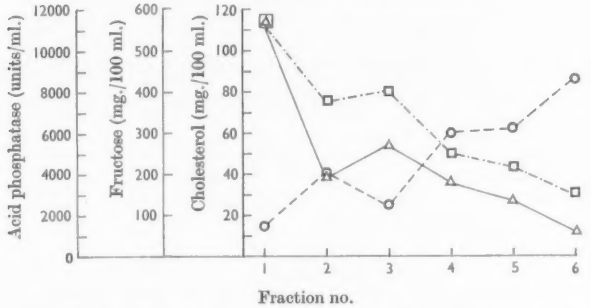

Fig. 1. Distribution of acid phosphatase (△), fructose (○) and cholesterol (□) in a 'split-ejaculate' from a healthy man. The pattern of distribution indicates that cholesterol is secreted from the same organ as acid phosphatase, i.e. the prostate gland.

are given in Table 1 and for one of the subjects in Fig. 1 also.

DISCUSSION

Little information is available on the lipid components in seminal plasma from various mammals. Scott (1945) found a mean content of total lipids corresponding to 186mg./100ml. of human seminal plasma. Cholesterol accounted for about 50–60% of this amount. Goldblatt (1935) found the average cholesterol concentration to be about 80mg./100ml. On the other hand, stallion semen had about the same concentration of lipids as human semen but the cholesterol content was only 4mg./100ml. (cf. Mann, 1964). For other mammals no data seem to be available.

Cholesterol originates most probably entirely from the prostate gland. This finding differs from the proposal by Scott (1945) that the fluid from the seminal vesicles should contain twice as much cholesterol as that from the prostate gland. This proposal was based on analyses of organ extracts.

About 40% of the cholesterol in semen occurred in the free form, a value that corresponds to that for human blood plasma. On the other hand, Scott (1945) reported that he found 90% of the cholesterol in the enlarged prostate gland to be in its free form. There seems to be no value available for the normal prostate gland.

It is known that the spermatozoa can metabolize fatty acids, but lipids themselves do not seem to influence the sperm metabolism (cf. Mann, 1964). It has been suggested that the lipoproteins in semen may act as protective colloids on the sperm cells. The possible significance of cholesterol for the metabolism and fertilizing properties of the spermatozoa is, however, not known.

Swyer (1942) observed that the adenomatous part of hypertrophic prostate glands contained about twice as much cholesterol as did the normal tissue. Nylander (1955) found that the lipid pattern of the semen showed characteristic changes in samples obtained from patients with prostate hypertrophia. It therefore appears that studies on the biochemical composition of human semen may not only be of interest in relation to the reproduction but also of value in diagnosis and evaluation of the pathophysiology of diseases in the male accessory genital glands.

This study is part of a research programme supported by grants from the Swedish Medical Research Council (Project no. U238, W312) and the Population Council (Project no. M6356, M6472). The author also gratefully acknowledges the technical assistance of Miss Solbritt Eldwinger, Miss Brittmarie Magnusson and Mrs Barbro Luttropp. Semen samples were generously supplied from the Sterility Laboratory of the Karolinska Hospital (head Dr E. Nordlander).

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Influence of the Metabolism of Ethanol on the Lactate/Pyruvate Ratio of Rat-Liver Slices

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(*Received* 23 *April* 1965)

1. The influence of ethanol on the redox level of the redox pair lactate/pyruvate has been studied in experiments with rat-liver slices. 2. Ethanol had no effect on oxygen consumption but strongly depressed carbon dioxide formation. On the assumption that ethanol is oxidized to acetate in the liver slices, it could be calculated that most of the oxygen that disappeared was consumed in this reaction. 3. Addition of ethanol to the incubation medium increased the lactate/pyruvate ratio and when all the ethanol had been oxidized the redox value decreased to the normal again. Ethanol depressed the pyruvate concentration, whereas the lactate concentration was not much influenced. 4. Acetaldehyde in the concentrations present during ethanol oxidation did not influence the lactate/pyruvate ratio. Higher concentrations, however, increased the redox state. 5. Acetate in the concentrations present during ethanol oxidation in the experiments, and also in higher concentrations, did not influence the lactate/pyruvate ratio. 6. The mechanism by which ethanol influences the lactate/pyruvate ratio is discussed.

Ethanol has no appreciable effect on the oxygen consumption of man (Lundquist, Tygstrup, Winkler, Mellemgaard & Munck-Petersen, 1962*b*) or of the perfused rat liver (Forsander, Räihä, Salaspuro & Mäenpää, 1965). The redox state of the liver is shifted towards a more reduced state, however, when ethanol is oxidized. It has been shown that the ratio of the substrate pairs lactate/pyruvate and β-hydroxybutyrate/acetoacetate in the blood increases during ethanol oxidation. This takes place both in man (Büttner, 1961; Lundquist *et al.* 1962*b*) and in the perfused rat liver (Forsander *et al.* 1965). It has also been found that the ratio between the reduced and oxidized NAD of the rat liver changes towards a more reduced state when ethanol is oxidized (Smith & Newman, 1959; Räihä & Oura, 1962).

Ethanol is broken down in the liver in two stages, first by oxidation of ethanol to acetaldehyde and then by further oxidation of this compound to acetate. These two reactions seem to be catalysed only by NAD-dependent dehydrogenases (Lundquist, Fugmann, Rasmussen & Svendsen, 1962*a*). The concentration of acetaldehyde in the blood is normally low but rises slightly during ethanol oxidation (Büttner, 1961). In the intact organism the acetate formed from ethanol is carried away from the liver by the blood stream and oxidized further extrahepatically (Forsander, Räihä & Suomalainen, 1960; Lundquist *et al.* 1962*b*). In experiments with perfused liver, liver slices or suspensions, however, acetate accumulates in the medium and only a small part is broken down to carbon dioxide (Leloir & Munoz, 1938; Forsander *et al.* 1960; Lundquist *et al.* 1962*b*). The present investigation is a study of whether the influence on the lactate/pyruvate redox value during ethanol oxidation is produced by ethanol or by its breakdown products acetaldehyde or acetate.

MATERIAL AND METHODS

Albino rats of Wistar origin and of both sexes, weighing between 200 and 300 g., were used for the experiments. Before the investigation the animals were kept on an ordinary laboratory diet available *ad lib.* The rats were killed by decapitation and bled, and the livers quickly removed and chilled in ice-cold saline. Liver slices were prepared with a Stadie–Riggs hand microtome.

The oxygen consumption and carbon dioxide production of the liver slices were measured in a Warburg apparatus by the technique of F. Dickens and F. Simer (Dixon, 1951). Krebs–Ringer bicarbonate buffer was used as medium (Umbreit, Burris & Stauffer, 1964). The respiratory quotient (R.Q.) and the total amounts of acids formed were calculated from the results of the experiments. The flasks containing the medium and liver slices (9:1, unless otherwise stated) were filled with oxygen–carbon dioxide (95:5) and shaken mechanically at 37°. The ethanol content of the medium was estimated enzymically by the method described by Bücher & Redetzki (1951). Lactate and pyruvate were determined enzymically with test kits supplied by C. F. Boehringer und Soehne G.m.b.H., Mannheim.

The reagents used were all analytically pure grade and

Table 1. *Oxygen consumption, carbon dioxide formation, respiratory quotient, acid production and ethanol oxidation of rat-liver slices*

The slices (200 mg.) were incubated in 2 ml. of Krebs–Ringer bicarbonate buffer for 60 min. at 37°. Experimental details are given in the text. Ethanol oxidation was analysed in different experiments, with identical incubation conditions. Ethanol to a final concentration of 17 mM was added to the flasks indicated. Results are given as the mean ± S.E.M. with the number of observations in parentheses.

	O_2 (μmoles/g. wet wt./min.)	CO_2 (μmoles/g. wet wt./min.)	R.Q.	Acid formation (μmoles/g. wet wt./min.)	Ethanol oxidation (μmoles/g. wet wt./min.)
Without ethanol	−1·64 ± 0·08 (16)	1·21 ± 0·07 (16)	0·74	0·51 ± 0·02 (16)	—
With ethanol	−1·63 ± 0·12 (16)	0·03 ± 0·08 (16)	0·02	1·37 ± 0·07 (16)	1·38 ± 0·02 (24)

the acetaldehyde was freshly distilled before use. The acetate used was as a sodium salt.

RESULTS

Ethanol had no effect on the oxygen consumption of the liver slices (Table 1). The carbon dioxide production was greatly depressed, however, by addition of ethanol to the medium. The R.Q. then decreased from the normal value of 0·74 to 0·02. As was to be expected, the production of acids was much greater in the experiments in which ethanol had been added to the medium than in the control experiments owing, presumably, to acetic acid production. The oxidation of ethanol was of the same order as the oxygen consumption.

During the preparation of the liver slices the tissue was in an anoxic state and 15 min. after incubation of the liver slices the lactate/pyruvate ratios were between 27 and 34 (Fig. 1). At this time ethanol was added to every second incubation flask and this produced a great increase in the ratio of this substrate pair. In the control flasks without addition of ethanol the ratio after 60 min. incubation was 12 and stayed at this value until the end of the experiment. After 105 min. incubation most of the ethanol added to the experimental flasks had disappeared and the lactate/pyruvate ratio had decreased, and after 150 min. was almost the same as in the control experiments. The lactate content of the medium increased at the beginning of the experiment but after 60 min. incubation reached a rather steady value (Table 2). The concentration of lactate and of pyruvate rose to about the same value as that given by Hohorst, Kreutz & Bücher (1959) for the intact rat liver. The shift of the lactate/pyruvate ratio with ethanol was mainly due to a decrease in the concentration of pyruvate. The lactate was not so much influenced. In this experiment ethanol had a slight effect on the lactate–pyruvate pool but this effect seems to depend on the nutritional condition of the liver. In a 'fasted' liver ethanol increases the pool (Forsander *et al.* 1965).

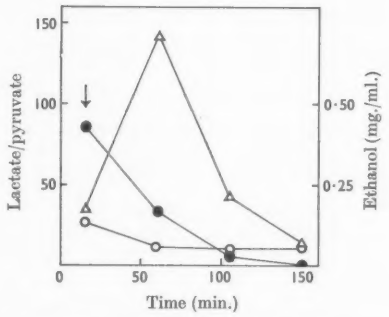

Fig. 1. Lactate/pyruvate ratio and ethanol concentration of the medium at different times during incubation of 2·0 g. of liver slices at 37° in 25 ml. of Krebs–Ringer bicarbonate buffer containing 11 mM-glucose. Ethanol (0·17 m-mole) was added at 15 min. to one series of flasks. Experiments ○, without ethanol, △, with ethanol (●, ethanol concentration).

Acetaldehyde is reported to be oxidized in the rat liver at a rate of about 66 μmoles/g. of liver/hr. (Lundquist *et al.* 1962*a*). With optimum substrate concentration this would mean that the acetaldehyde concentration in the experiment described in Table 3 would decrease by 3·3 mM. The concentration of 1·78 mM used in one of the experiments should therefore decrease to about zero during the experiment. This concentration, however, increased the lactate/pyruvate ratio somewhat and higher concentrations increased the ratio further. The acetaldehyde had a greater effect on the pyruvate than on the lactate concentrations. With an acetaldehyde concentration of 17·8 mM a big increase in the ratio was observed. The total lactate–pyruvate pool was decreased by acetaldehyde.

Acetate in concentrations between 1·0 and 10·0 mM had no appreciable effect on the lactate/pyruvate ratio (Table 4). With the higher acetate

Table 2. *Lactate and pyruvate concentrations of the medium at different times*

Liver slices (2·0 g.) were incubated in 25 ml. of Krebs–Ringer bicarbonate buffer at 37°, containing 11 μmoles of glucose/ml. Ethanol (0·17 m-mole) was added to one series of flasks after 15 min. Values given are means ± S.E.M. for three experiments.

Sampling time (min.).....	15	60	105	150
Lactate (μmoles/ml. of incubation mixture)				
without ethanol	0·586 ± 0·050	1·300 ± 0·044	1·694 ± 0·228	1·762 ± 0·210
with ethanol	0·660 ± 0·088	1·196 ± 0·126	1·104 ± 0·116	1·584 ± 0·010
Pyruvate (μmole/ml. of incubation mixture)				
without ethanol	0·022 ± 0·008	0·114 ± 0·032	0·163 ± 0·026	0·160 ± 0·017
with ethanol	0·019 ± 0·007	0·008 ± 0·004	0·026 ± 0·014	0·109 ± 0·003
Lactate + pyruvate (μmoles/ml. of incubation mixture)				
without ethanol	0·608 ± 0·044	1·414 ± 0·052	1·857 ± 0·250	1·922 ± 0·228
with ethanol	0·679 ± 0·084	1·204 ± 0·130	1·130 ± 0·104	1·693 ± 0·012

Table 3. *Influence of acetaldehyde on the lactate and pyruvate concentrations*

Liver slices (500 mg.) were incubated for 60 min. at 37° in 5 ml. of Krebs–Ringer bicarbonate buffer containing 11 μmoles of glucose/ml. and different concentrations of acetaldehyde as indicated. Results are given as the means ± S.E.M. with the number of observations in parentheses.

Acetaldehyde (μmoles/ml. of incubation mixture)	Lactate (μmoles/ml. of incubation mixture)	Pyruvate (μmole/ml. of incubation mixture)	Lactate + pyruvate (μmoles/ml. of incubation mixture)	Ratio lactate/ pyruvate
0	1·480 ± 0·100 (8)	0·106 ± 0·010 (8)	1·586 ± 0·108 (8)	13·1
1·78	1·134 ± 0·036 (3)	0·054 ± 0·018 (3)	1·188 ± 0·051 (3)	19·0
8·90	1·152 ± 0·050 (3)	0·044 ± 0·031 (3)	1·196 ± 0·018 (3)	24·0
17·8	0·978 ± 0·054 (8)	0·008 ± 0·002 (8)	0·986 ± 0·052 (8)	119·0

Table 4. *Influence of acetate on the lactate and pyruvate concentrations*

Liver slices (500 mg.) were incubated for 60 min. at 37° in 5 ml. of Krebs–Ringer bicarbonate buffer containing 11 μmoles of glucose/ml. and different concentrations of acetate. Results are expressed as the means ± S.E.M. with the number of observations in parentheses.

Acetate (μmoles/ml. of incubation medium)	Lactate (μmoles/ml. of incubation medium)	Pyruvate (μmole/ml. of incubation medium)	Lactate + pyruvate (μmoles/ml. of incubation medium)	Ratio lactate/ pyruvate
0	0·924 ± 0·156 (6)	0·087 ± 0·018 (6)	1·011 ± 0·168 (6)	10·7
1·0	1·062 ± 0·104 (6)	0·089 ± 0·008 (6)	1·151 ± 0·112 (6)	12·0
4·0	0·916 ± 0·088 (5)	0·079 ± 0·008 (5)	0·995 ± 0·091 (5)	11·6
10·0	0·796 ± 0·162 (5)	0·061 ± 0·008 (5)	0·857 ± 0·167 (5)	13·5

concentrations a slight decrease in the lactate–pyruvate pool could be observed.

DISCUSSION

The concentrations of lactate and pyruvate in the circulating blood are in equilibrium with those of the cytoplasmic compartment of the liver and the ratio between this substrate pair is the same inside the liver and in the blood (Hohorst *et al.* 1959). In the liver cytoplasm the substrate pair is reported to be in redox equilibrium with the other NAD-dependent redox pairs (Hohorst *et al.* 1959). When rat-liver slices were incubated in the Krebs–Ringer bicarbonate buffer the lactate and pyruvate contents of the medium rose (Table 2) until a value was reached which was approximately the same as that reported for intact rat-liver tissue (Hohorst

et al. 1959). The lactate/pyruvate ratio was also identical with that for the liver. It can therefore be assumed that, at least in respect of this substrate pair, the incubation medium acts as an extended cytoplasmic compartment.

In man the arterial blood lactate/pyruvate ratio has been found to rise to about two to three times the normal value when ethanol has been consumed (Büttner, 1961). In rat-liver perfusion experiments the ratio may increase eight- to nine-fold. In the experiments with liver slices (Fig. 1) ethanol raised the ratio to about 12-fold that of the controls.

The redox level of the lactate/pyruvate substrate pair of the liver has been found to be influenced by inadequate oxygen supply (Schimassek, 1962*a*,*b*), by starvation and by some hormones (Hohorst, Kreutz, Reim & Hübener, 1961). Since the oxygen consumption is not depressed by ethanol, it cannot be assumed that lack of oxygen is the cause of the shift in the redox value (Table 1). The effects of starvation and of hormones can also be excluded in these experiments *in vitro*.

It has been shown that acetaldehyde inhibits the mitochondrial oxidation of certain substrates (Kiessling, 1963). If in fact it does retard the reoxidation of a reduced intermediate component carrying hydrogen from the cytoplasm to the mitochondria it is understandable that the oxidation/reduction state of the cytoplasm will change. During ethanol oxidation the concentration of acetaldehyde is very low, less than 10μM (Lundquist *et al.* 1962*b*), and therefore the aldehyde cannot be the cause of the increased redox ratio. Since the shift in the lactate/pyruvate ratio is not influenced by acetate, either, which is the end product of ethanol breakdown in the liver, the breakdown of ethanol to acetaldehyde must be the cause of the shifted redox state.

When ethanol is present in the medium the oxidation of the substrate to acetate will almost completely monopolize the oxygen consumption (Table 1). In earlier experiments Leloir & Munoz (1938) and Lundquist *et al.* (1962*b*) have found that about 75% of the oxygen consumed is utilized for the oxidation of ethanol to acetate. This means that a big shift has taken place in the metabolic pathways. Fritz (1961) has calculated that the liver utilizes 77% of the oxygen for the breakdown of fatty acids to carbon dioxide and ketone bodies, whereas the rest is consumed in several other minor oxidation reactions. The fatty acids are oxidized in the mitochondria and hence the extramitochondrial oxygen consumption is low during normal conditions. When ethanol is oxidized, the oxidation of fatty acid is totally inhibited, which can be seen from the fact that no carbon dioxide is produced and also that ketone-body production is not appreciably influenced by ethanol (Lundquist *et al.* 1962*a*; Forsander *et al.* 1965). By contrast, the extramitochondrial oxidation increases since the alcohol dehydrogenase, which oxidizes ethanol to acetaldehyde, is mainly located in the cytoplasm of the liver cell (Nyberg, Schuberth & Änggård, 1953; Räihä & Koskinen, 1964). The rate of oxidation of ethanol by alcohol dehydrogenase seems to be faster than the regulated reoxidation of NADH in the respiratory chain of the mitochondria. This would explain why the NADH/NAD^+ ratio of the cytoplasm increases during ethanol oxidation, an increase which can also be seen in the increased lactate/pyruvate ratio.

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5-Dehydroshikimate Reductase in the Tea Plant (*Camellia sinensis* L.)

PROPERTIES AND DISTRIBUTION

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(*Received* 28 *June* 1965)

1. A method for the extraction of 5-dehydroshikimate reductase (EC 1.1.1.25) from tea plant tissues in an active soluble state has been developed. It is dependent on the use, in the extraction medium, of an insoluble polyphenol adsorbent (Polyclar AT), which prevents the polyphenols present from precipitating all the proteins. 2. The enzyme has the following properties: pH optima at pH10·1 in glycine–sodium hydroxide buffer and at pH 7·7 in tris–hydrochloric acid buffer; K_m (NADP) 32μM and K_m (shikimate) 0·43mM; and NADP-specificity. It was completely inhibited by 0·33mM-*p*-chloromercuribenzoate and this inhibition was completely reversed by 10mM-cysteine. Iodoacetate and arsenite inhibited the enzyme to a smaller extent. 3. The specific activity of the enzyme was higher in the parts of the actively growing shoot tips (third leaf > stem > second leaf > first leaf > bud) than in the mature leaves. However, the mature leaves had the greatest total activity. 4. The importance of these findings with respect to flavanol biosynthesis in tea plants is discussed.

The shikimic acid pathway (Neish, 1960, 1964) is the sole route by which aromatic compounds are formed in *Escherichia coli* (Davis, 1955). However, in higher plants the importance of this pathway in the biosynthesis of aromatic compounds is not yet clear. Shikimic acid has been found in many plants and it is a good precursor of a wide variety of aromatic compounds in higher plants, but there is also evidence for the operation of other pathways in these organisms (Neish, 1960, 1964). Enzymic studies should help to clarify this point, but very few reports on this subject have appeared (Conn, 1964).

Plant tissues that would presumably contain the highest concentration of the enzymes involved in the biosynthesis of aromatic compounds, namely those containing high concentrations of these compounds, are the most refractory for enzyme studies because of the protein-denaturing properties of such compounds (Sanderson, 1965*a*). However, techniques have recently been developed that enable soluble enzymes to be extracted from such tissues (Loomis & Battaile, 1964; Sanderson, 1964; Badran & Jones, 1965) and these techniques have now been applied to extraction of enzymes of the shikimic acid pathway from tea plants.

Flavonoid compounds usually comprise 20–35% of the dry weight of actively growing shoot tips of the tea plant (*Camellia sinensis* L.) (Vuataz, Brandenberger & Egli, 1959) and they are of great economic importance in these tissues because of their dominant role in the manufacture of black tea (Roberts, 1962; Sanderson, 1965*b*). The possible importance of shikimic acid in the biosynthesis of flavanols in tea plants has been shown with [^{14}C]shikimic acid (Zaprometov, 1962; Zaprometov & Bukhlaeva, 1963), but enzymic studies would be useful for a fuller understanding of the biosynthesis of these compounds in these plants. In the present paper the occurrence, properties and distribution of 5-dehydroshikimate reductase (EC 1.1.1.25) in tea plants are described.

MATERIALS AND METHODS

Chemicals. NAD, NADP, $NADH_2$, $NADPH_2$ and tris were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. Shikimic acid, D-catechin and glycine were purchased from Calbiochem A.-G., Lucerne, Switzerland. Polyclar AT (an insoluble cross-linked polyvinylpyrrolidone) was purchased from Antara Chemicals Division of General Aniline and Film Corp., New York, N.Y., U.S.A. All other chemicals were purchased from British Drug Houses Ltd., Poole, Dorset.

Source of plant material. This was taken from blocks of clonal tea growing in fields adjoining this Laboratory (1500m. elevation). Rapidly growing shoot tips, comprising the bud, first two leaves and the included stem, from mature plants in plucking (cf. Eden, 1958; Harler, 1963) of clone TRI.777 were used in all experiments except as otherwise noted.

Preparation of enzyme extracts. Fresh shoot tips (5·0g.) were frozen at −15° for 1hr. The frozen shoot tips were ground with 3·0g. of Polyclar AT, about 2·5g. of acid-

washed sand and 50ml. of 0·1M-sodium phosphate buffer, pH7·0. The homogenate was sieved through muslin cloth and the filtrate was centrifuged at 3000*g* for 20min. The supernatant solution was used as enzyme preparation. All these operations were carried out in a cold room at 4°.

Assay of 5-dehydroshikimate reductase. The assay was carried out as described by Balinsky & Davies (1961*a*) by spectrophotometrically following the increase in extinction at 340mμ due to the production of $NADH_2$ according to the following reaction:

$$\text{Shikimate} + \text{NADP} \longrightarrow \text{5-dehydroshikimate} + \text{NADPH}_2$$

The standard assay was run in silica cuvettes with a 1·0cm. light-path at room temperature (about 22°). The reaction mixture contained shikimic acid (1·0mM) and NADP (170μM) in 2·5ml. of 0·1M-glycine–NaOH buffer, pH10·1, 0·1ml. of enzyme preparation (0·08–0·11mg. of protein) and 0·4ml. of water.

Protein determination. Protein was precipitated at 4° with 5% (w/v) trichloroacetic acid overnight. The precipitate was collected, washed with 5% (w/v) trichloroacetic acid, and nitrogen was determined by a micro-Kjeldahl procedure (Bailey, 1962). The factor 6·25 was used to convert nitrogen content into protein content.

Flavanol determination. Flavanols were determined by the diazotized sulphanilic acid method described by Nakagawa & Torii (1964). D-Catechin was used as standard.

Units of enzyme activity and calculations. One unit of activity is defined as that amount of enzyme catalysing the oxidation of 1μmole of shikimic acid (or the reduction of 1μmole of NADP)/min. Specific activity is defined as units of activity/mg. of protein (*Report of the Commission on Enzymes of I.U.B.*, 1961). A value of $6{\cdot}22 \times 10^6$ (Horecker & Kornberg, 1948) for the millimolar extinction coefficient, ϵ_{mM}, was used to convert E_{340} readings into $NADPH_2$ concentrations.

RESULTS

Effect of the amount of Polyclar AT on the extraction of soluble 5-dehydroshikimate reductase from tea shoot tips. Shoot tips were homogenized together with various proportions of Polyclar AT to determine the optimum amount for the extraction of soluble enzyme. Maximum activity was obtained with 0·6g. of Polyclar AT/g. fresh wt. of tissue. This coincided with the amount of Polyclar AT at which maximum protein and minimum flavanols were extracted (Table 1).

All enzyme preparations contained some flavanols (Table 1), although when 0·6g. or more of Polyclar AT/g. fresh wt. of tissue was used the concentration of flavanols was very low. Balinsky & Davies (1961*b*) have reported that polyphenolic compounds similar to those occurring in tea shoot tips (Roberts, 1962) inhibit the 5-dehydroshikimate reductase extracted from peas, so it was desirable to determine the effect of removing these residual polyphenols from the preparations. Accordingly, the original extracts were treated with additional amounts of Polyclar AT as explained in Table 1. This second treatment with Polyclar AT was effective in removing residual flavanols without affecting the protein concentration in these extracts. Further, the enzyme activity was increased in the two extracts containing significant amounts of both protein and flavanols, namely the extracts made with 0·2 and 0·4g. of Polyclar AT/g. fresh wt. of tissue. After removing the enzyme-inhibiting polyphenols the specific activities of all active

Table 1. *Effect of Polyclar AT on the extraction of soluble 5-dehydroshikimate reductase from tea shoot tips*

Extraction of tea shoot tips was carried out as described in the Materials and Methods section except that the amount of Polyclar AT in the extraction medium was varied as shown below. These extracts are the original extracts. Portions of the original extracts were treated with additional Polyclar AT for 30min. at 4°. After this treatment these latter solutions were centrifuged at 3000*g* for 20min. at 4° and the supernatants are denoted as Polyclar-treated extracts. All assays and analyses were carried out as described in the Materials and Methods section.

Amount of Polyclar AT in extraction medium (g./g. fresh wt. of tissue)	Amount of Polyclar AT used to treat original extracts (g./g. fresh wt. of tissue)	Protein (mg./ml.)		Flavanols (μg./ml.)		Total activity (unit/ml.)		Specific activity (unit/mg. of protein)	
		Original extract	Polyclar-treated extract	Original extract	Polyclar-treated extract	Original extract	Polyclar-treated extract	Original extract	Polyclar-treated extract
0	0·5	0·01	0·01	2270	11	—	0	—	0
0·1	0·4	0·05	0·05	525	0	0	0	0	0
0·2	0·3	0·36	0·36	110	0	0	0·05	0	0·14
0·4	0·1	0·84	0·84	41	0	0·10	0·13	0·12	0·15
0·6	0·1	1·06	1·06	23	0	0·16	0·15	0·15	0·14
0·8	0·1	1·21	1·21	24	0	0·15	0·16	0·12	0·13
1·0	0·1	1·26	1·26	22	0	0·16	0·15	0·13	0·12

preparations were the same except those made with large amounts (more than 0·6g./g. fresh wt. of tissue) of Polyclar AT. This decrease in specific activity when large amounts of Polyclar AT are used has been noted repeatedly but the cause is not known.

These results generally agree with those obtained previously for Polycaprolaktam powder in the extraction of soluble catechol oxidase from these same tissues (Sanderson, 1964), except that Polyclar AT is about twice as effective on a dry-weight basis as Polycaprolaktam in adsorbing polyphenolic compounds and thereby preventing them from precipitating the proteins.

Effect of time and enzyme concentration. Experiment showed that ΔE_{340} was proportional with time up to at least 7min. and with enzyme concentration up to at least 65μg. of protein/ml. of reaction mixture under the conditions of the standard assay described in the Materials and Methods section. There was no reaction when the enzyme preparation was heated in a boiling-water bath for 3min. before the assay.

Requirement for substrate and cofactors: activity of related enzymes. When shikimate or NADP was omitted from the standard reaction mixture, no change in E_{340} occurred. Also, no change in E_{340} occurred when quinate was substituted for shikimate in the presence of either NAD or NADP. Neither $NADH_2$ nor $NADPH_2$ was oxidized by these enzyme preparations when they were substituted for NADP in the standard reaction mixture.

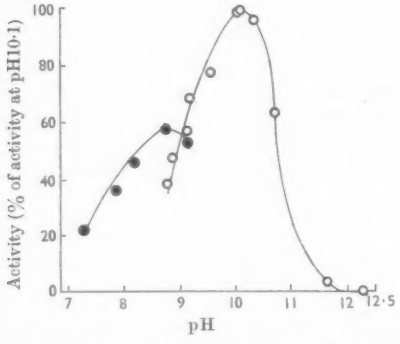

Fig. 1. Effect of pH and buffer composition on 5-dehydroshikimate-reductase activity. Enzyme activity was determined as described in the Materials and Methods section except that the buffer used in the reaction mixture was varied as shown: ●, 0·1M-tris–HCl buffers; ○, 0·1M-glycine–NaOH buffers. The enzyme preparation contained 0·94mg. of protein/ml. and its specific activity was 0·15unit/mg. of protein at pH10·1.

Effect of pH *and buffer composition.* The standard assay was modified by substituting 0·1M-glycine–sodium hydroxide or 0·1M-tris–hydrochloric acid buffers at various pH values for the 0·1M-glycine–sodium hydroxide buffer, pH10·1, normally used. A sharp pH optimum occurs at pH10·1 in 0·1M-glycine–sodium hydroxide buffer (Fig. 1). However, in 0·1M-tris–hydrochloric acid buffer the pH optimum is at pH7·7.

Effect of changes in concentration of NADP and shikimic acid. The effects of varying separately the concentrations of NADP and shikimic acid in the

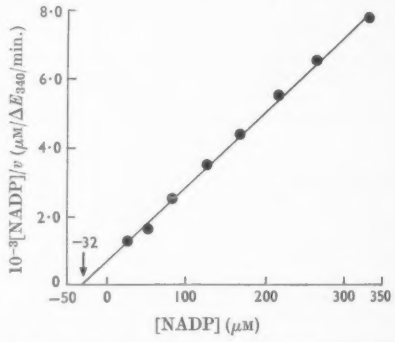

Fig. 2. Reciprocal plot of the effect of NADP concentration on 5-dehydroshikimate-reductase activity. Enzyme activity was determined as described in the Materials and Methods section except that the NADP concentration was varied as shown.

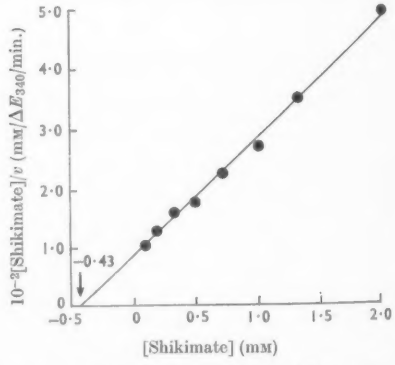

Fig. 3. Reciprocal plot of the effect of shikimate concentration on 5-dehydroshikimate-reductase activity. Enzyme activity was determined as described in the Materials and Methods section except that the shikimate concentration was varied as shown.

standard assay system were measured and the results are presented as reciprocal plots (Lineweaver & Burk, 1934) in Figs. 2 and 3. The K_m for NADP was 32 μM and K_m for shikimic acid was 0·43 mM.

Effect of various compounds. The effect of several compounds on 5-dehydroshikimate reductase is shown in Table 2. *p*-Chloromercuribenzoate was an effective inhibitor of the enzyme and this inhibition could be completely reversed by cysteine. These results suggest that the enzyme contains essential thiol groups. Two other reagents for thiol groups, namely arsenite and iodoacetate, inhibited the enzyme to a much smaller extent.

Distribution of 5-dehydroshikimate reductase in the tea plant. Mature tea plants in plucking (Eden, 1958; Harler, 1963) were sampled to determine the distribution of the enzyme. The following samples, which were taken simultaneously, were collected: shoot tips, which were dismembered into (1) buds, (2) first leaves, (3) second leaves, (4) third leaves and (5) stems; (6) maintenance leaves, which are the mature leaves in whose axils the shoot tips to be plucked arise; (7) 'old' leaves, which are mature leaves found within the tea bush below the plucking table. All samples were extracted as described in the Materials and Methods section by using 0·6 g. of Polyclar AT/g. fresh wt. of tissue.

Table 3 shows that all parts of the plant sampled had appreciable amounts of 5-dehydroshikimate-reductase activity. The specific activity in both samples of mature leaves was low, but because of their relatively large size they still contained more total activity than the parts of the shoot tips.

Table 2. *Effect of various compounds on 5-dehydroshikimate-reductase activity*

Assays were carried out as described in the Materials and Methods section. Compounds to be tested were incubated with the enzyme in the 0·1M-glycine–NaOH buffer, pH10·1, for 5 min. before adding shikimic acid and NADP.

Compound added	Final concn. in reaction mixture (mM)	Activity (% of original)
None	—	100
EDTA	5	115
Potassium cyanide	1	75
Cysteine	10	105
p-Chloromercuribenzoate	0·33	0
p-Chloromercuribenzoate + cysteine	0·33 + 10	100
Arsenite	1	75
Iodoacetate	1	70

DISCUSSION

5-Dehydroshikimate reductase has been extracted from *E. coli* (Yaniv & Gilvarg, 1955), etiolated pea epicotyls (Balinsky & Davies, 1961*a*) and mung-bean seedlings (Nandy & Ganguli, 1961). These enzymes were purified 9·5-, 68- and 6·5-fold, to obtain preparations with specific activities 0·08, 0·23 and 0·13 unit/mg. of protein respectively. The specific activity of the crude enzyme preparations obtained from tea shoot tips compared favourably with specific activities of the most pure of these earlier preparations (Table 4). Table 4 shows that the four enzymes are indeed very similar.

The distribution of 5-dehydroshikimate reductase in the tea plant is of particular interest because of its possible importance in the biosynthesis of flavonoid compounds. Zaprometov (1962) and Zaprometov & Bukhlaeva (1963) have shown that shikimic acid is readily incorporated into the flavanols of tea shoot tips in experiments with [^{14}C]shikimic acid. In addition, Zaprometov (1963)

Table 3. *Distribution of 5-dehydroshikimate reductase in the tea plant*

All methods used were as outlined in the Materials and Methods section. The samples are described in the text.

Part of plant sampled	Fresh wt. of part (mg.)	Total activity (units/g. fresh wt. of tissue)	Specific activity (unit/mg. of protein)	Total activity/plant part (% of activity in second leaf)
Buds	53	1·15	0·12	21
First leaves	88	1·51	0·15	46
Second leaves	166	1·73	0·18	100
Third leaves	212	1·74	0·27	129
Stems	150	1·04	0·22	54
Maintenance leaves	525	1·09	0·07	199
'Old' leaves	867	0·38	0·05	115

Table 4. *Comparison of the properties of 5-dehydroshikimate reductase extracted from* E. coli, *pea epicotyls, mung-bean seedlings and tea shoot tips*

Enzyme property	*E. coli* (Yaniv & Gilvarg, 1955)	Pea epicotyls (Balinsky & Davies, 1961*a*)	Mung-bean seedlings (Nandy & Ganguli, 1961)	Tea shoot tips (this paper)
Sp. activity in crude extracts	0·008	0·003	0·020	0·15–0·27
Sp. activity in purified preparation	0·078	0·23	0·13	—
pH optimum	8·5	10·0	8·0	10·1
K_m (NADP)	31 μM	7 μM	117 μM	32 μM
K_m (shikimate)	0·06 mM	0·23 mM	0·09 mM	0·43 mM
NADP-specificity	Yes	Yes	Yes	Yes
Inhibition by *p*-chloromercuribenzoate	—	92% by 2·5 mM	100% by unspecified concn.	100% by 0·33 mM
Inhibition by iodoacetate	—	16% by 10 mM	—	30% by 1 mM

has shown that photosynthetically assimilated $^{14}CO_2$ is in large part incorporated into flavanols in the young (first, second and third) leaves but only in small part in the old (fifth and sixth) leaves. The present findings support those of the Russian workers in two ways: (1) an enzyme required for the operation of the shikimic acid pathway in tea plants has been demonstrated; (2) variations in specific activity of 5-dehydroshikimate reductase in tea plants would be expected to produce the pattern of labelling from photosynthetically assimilated $^{14}CO_2$ that they found.

Now that a method for the extraction of active soluble enzymes from tissues containing high concentrations of protein-precipitating polyphenols has been developed, it should be possible to detect and assay other enzymes in tea plants and so to answer many of the questions about flavanol biosynthesis.

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Effect of Oxygen Pressure on Glycogen Synthesis by Rat-Liver Slices

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(*Received* 2 *July* 1965)

1. Glycogen synthesized by rat-liver slices 0·5mm. thick incubated at 1atm. oxygen pressure in Hastings medium with glucose was localized in the cells of the periphery of the slice. Cells of the interior of this slice do not synthesize glycogen. 2. Inner cells of thin slices (about 0·3mm. thick) can synthesize glycogen when such slices are incubated under the same conditions, but oxygen pressures higher than 1atm. are required if inner cells of slices 0·5mm. or more thick are to be able to synthesize glycogen. 3. Localization of newly synthesized glycogen in rat-liver slices incubated in Hastings medium with glucose does not depend on glucose concentration. 4. Calculation of the minimum oxygen pressure required to synthesize glycogen gives values between 0·09 and 0·17atm. 5. The advantages of high oxygen pressures for the study of the synthesis of glycogen and other compounds that require ATP are discussed.

Mammalian liver homogenates show a poor capacity to incorporate [^{14}C]glucose into glycogen (Figueroa, Pfeifer & Niemeyer, 1962; Banerjee & Ganguli, 1962), but liver slices incubated in Hastings medium show a higher rate of incorporation (Hastings, Teng, Nesbett & Sinex, 1952; Niemeyer & Figueroa, 1956; Cahill, Hastings, Ashmore & Zottu, 1958; Figueroa & Pfeifer, 1964). For this reason most of the studies of glycogen synthesis in mammalian liver have utilized the slice technique. Deane, Nesbett, Buchanan & Hastings (1947) showed that the cells in the slice are not uniform in their capacity to synthesize glycogen. The new glycogen was localized at the periphery of the slice whereas the cells of the interior did not exhibit glycogen synthesis. There was, in addition, disappearance of mitochondria at the interior of the slice. The localization of the new glycogen was attributed to an adequate supply of oxygen and nutrients in the cells located nearer the surface of the slice.

The present work deals with the effect of oxygen pressure on the localization of glycogen synthesized by rat-liver slices. It is shown that the amount of glycogen is directly proportional to the oxygen pressure, and that with higher-than-normal oxygen pressures the inner cells of the slice behave similarly to the cells of the periphery.

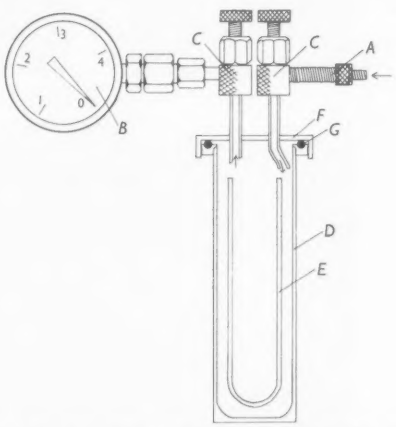

Fig. 1. Sealed cylinder used for the incubation of slices at high O_2 pressures. *A*, Valve of bicycle tyre where gas is introduced; *B*, manometer; *C*, valves that isolate the cylinder from the manometer and valve *A*; *D*, brass cylinder; *E*, glass flask where slices are incubated; *F*, screwed cover; *G*, rubber rim that permits the cover to close hermetically. The arrows indicate the direction of gas flow.

EXPERIMENTAL

Animals. Male albino rats from our Institute strain, weighing about 200g., were used. They were killed by decapitation after 24hr. without food but with free access to water. The liver was quickly excised and put in ice-cold 0·154M-KCl.

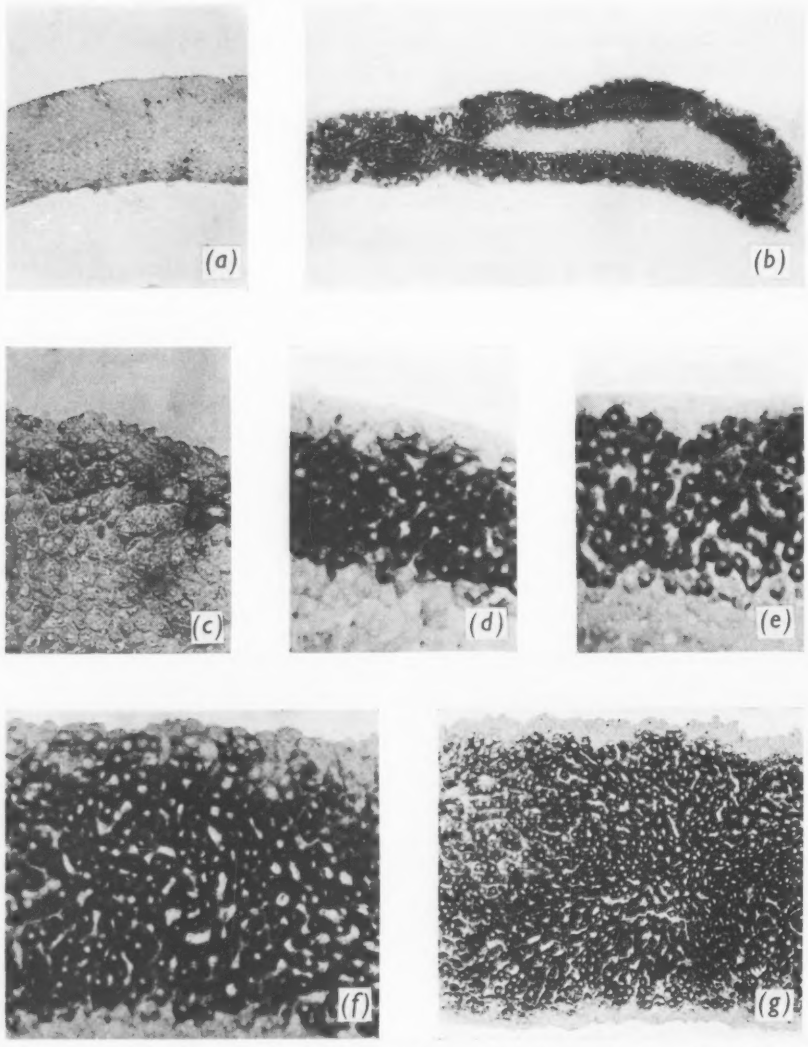

Fig. 2. Transverse sections of rat-liver slices fixed and stained for glycogen by the periodate–Schiff technique. All of them, except (*a*), were incubated for 45 min. in Hastings medium containing glucose (0·03 M) and with different O_2 pressures. (*a*) Non-incubated liver slice (glycogen content, 0·10%); magnification ×32. (*b*) Slice incubated with O_2 at 0·84 atm. (glycogen content, 0·62%); glycogen is absent in the interior of the slice at the right-hand side, which is a thick part; the left-hand side, which is thinner, is full of glycogen; magnification ×32. (*c*) Slice incubated with O_2 at 0·18 atm. (glycogen content, 0·12%); glycogen is confined to a rim about 0·08 mm. deep; cells along the very edge contain no glycogen, probably owing to damage; magnification ×180. (*d*) Slice incubated with O_2 at 0·50 atm. (glycogen content, 0·22%); glycogen occurs in a rim about 0·16 mm. deep; magnification ×180. (*e*) Slice incubated with O_2 at 0·84 atm. (glycogen content, 0·34%); the depth of glycogen reaches about 0·18 mm.; magnification ×180. (*f*) Slice incubated with O_2 at 1·39 atm. (glycogen content, 0·58%); the depth of glycogen reaches about 0·28 mm.; magnification ×180. (*g*) Slice incubated with O_2 at 2·7 atm. (glycogen content, 0·85%); the slice is 0·52 mm. thick; magnification ×90.

Incubation of the slices. Liver slices were made free-hand with a razor blade and put in an oxygenated and cold solution of 0·154 M-KCl. The slices were incubated in 25 ml. flasks by using the bicarbonate medium I of Hastings *et al.* (1952). Glucose was added to give a final concentration of 0·03 M unless otherwise stated. The medium was equilibrated with various gas mixtures. For incubations at pressures over 1 atm. a sealed cylinder (described below) was used. The flasks were put in a water bath at 37° for 45 min. At the beginning and at the end of the incubation period two flasks were removed, one for chemical determination of glycogen and the other one for histological examination.

Gas mixtures. (*a*) An O_2+CO_2 (95:5) mixture passed through water. The mixture had a total pressure of about 720 mm. Hg (average atmospheric pressure in Santiago). The partial pressure of O_2 was calculated to be 0·84 atm. by subtracting the partial pressure of water vapour at 37° and the pressure of CO_2.

(*b*) An $O_2+N_2+CO_2$ (20:75:5) mixture passed through water. This gives an O_2 pressure of 0·18 atm.

(*c*) An $O_2+N_2+CO_2$ (59:36:5) mixture passed through water. This gives an O_2 pressure of 0·50 atm.

(*d*) Several mixtures with O_2 pressures over 1 atm. The cylinder used for this kind of experiment was washed with an O_2+CO_2 (95:5) mixture at atmospheric pressure and then it was injected with pure O_2 to increase the pressure to that desired.

Apparatus used to incubate slices at pressures higher than 1 atm. For incubation experiments at pressures above atmospheric pressure the flask containing slices was placed in a brass cylinder that could be sealed hermetically. This cylinder was equipped with a pressure gauge to indicate the internal pressure and a valve of a bicycle tyre through which the gas entered. The changes in pressure were achieved by means of a bicycle pump arranged in such a manner that O_2 or other gas could be pumped into the sealed cylinder. Two internal valves isolated the interior from the manometer and the tyre valve to prevent any leakage of gas. To wash the cylinder with gas at atmospheric pressure the manometer and the tyre valve were loosened. A schematic representation of the apparatus is given in Fig. 1.

Glycogen determination. Glycogen was isolated by digesting the slices with 1 ml. of 30% (w/v) KOH in a boiling-water bath for 30 min. Then 0·1 ml. of 2% (w/v) Na_2SO_4 and 3 vol. of ethanol were added to precipitate the glycogen. It was left standing for at least 2 hr. in the cold room and centrifuged down, dissolved in water and precipitated again with ethanol. The isolated glycogen was determined by the method of Montgomery (1957).

Histological methods. For histological examination, slices of one of the flasks of each experimental condition were fixed in a solution containing 80 ml. of ethanol saturated with picric acid, 10 ml. of 40% formalin and 10 ml. of ethanol. It was left standing at 2–3° for 2 hr. and then 2 hr. at room temperature. The fixed slices were embedded in paraffin wax and sectioned transversely at 6 μ. Glycogen was detected by the periodate–Schiff method (Pearse, 1961). In some cases a study of mitochondria was made. The tissue slice was fixed in Zenker formol fluid for 24 hr., post-chromated in 3% (w/v) $K_2Cr_2O_7$ at 37° for 3 days, washed and embedded in paraffin wax to be sectioned and stained by the Mallory's ferric haematoxylin method (Langeron, 1949).

RESULTS

Figs. 2(*a*) and 2(*b*) show transverse sections of liver slices before and after incubation in Hastings medium with gas phase oxygen + carbon dioxide (95:5). This corresponds to oxygen at 0·84 atm. In confirmation of data of Deane *et al.* (1947), glycogen synthesis is limited to a rim nine or ten cells deep in the periphery of the slice. Inner cells of slices 0·5 mm. thick, corresponding approximately to 30 rows of cells, i.e. the optimum thickness recommended by Fuhrman & Field (1945) for the study of oxygen uptake, have no capacity to synthesize glycogen.

If thinner slices are used (about 20 rows of cells)

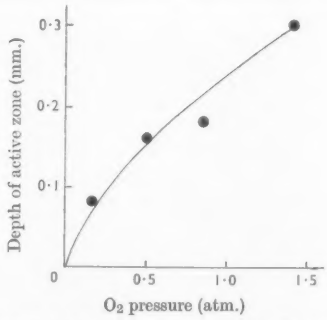

Fig. 3. Effect of O_2 pressure on the depth of the peripheral rim of liver slices that are able to synthesize glycogen when incubated with glucose in Hastings medium. Experimental details are given in the text.

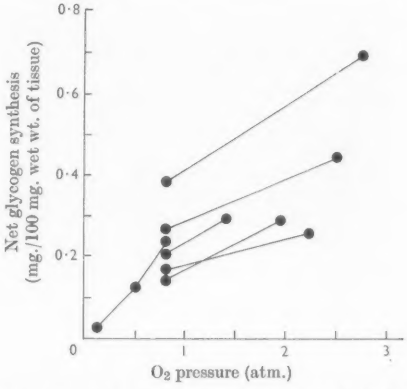

Fig. 4. Effect of O_2 pressure on glycogen synthesis of rat-liver slices incubated for 45 min. in Hastings medium. Points linked by a line correspond to slices from the same liver. Other experimental details are given in the text.

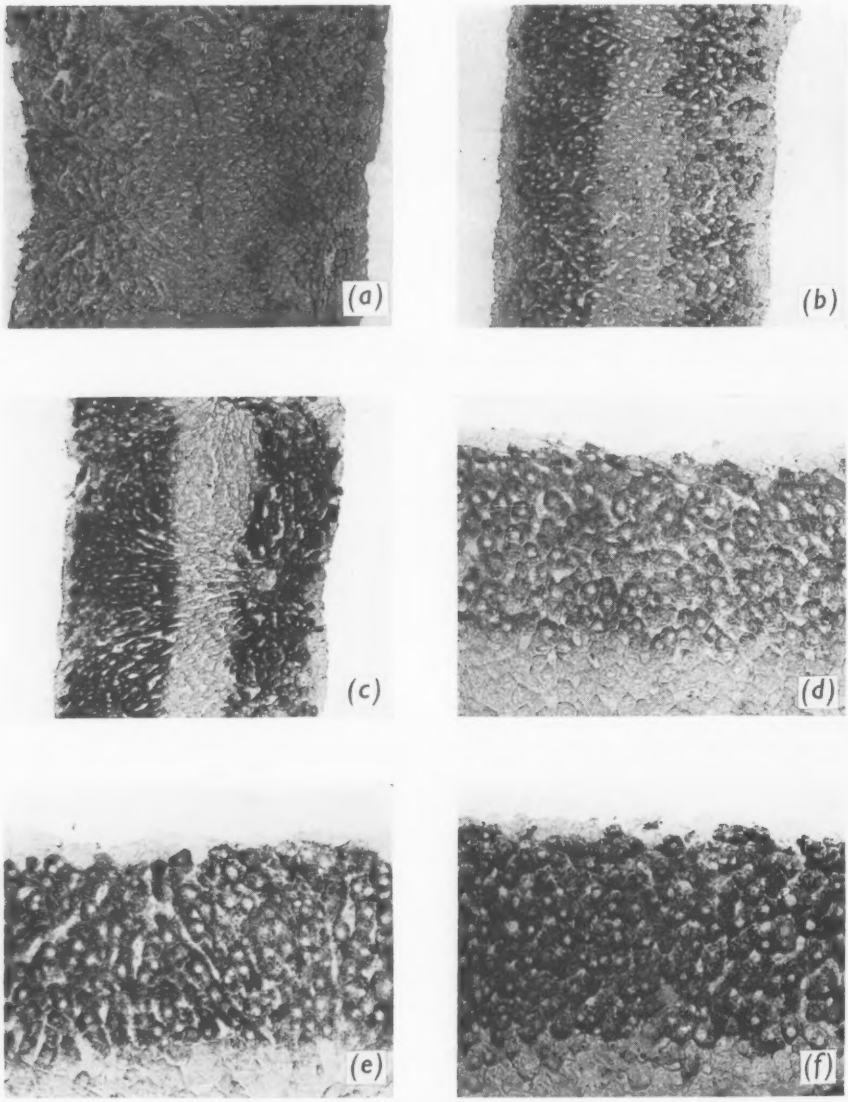

Fig. 5. Transverse sections of rat-liver slices fixed and stained for glycogen by the periodate–Schiff technique. They were incubated in Hastings medium with O_2 at 0·84atm. (*a*) Slice incubated with glucose (0·03M) for 10min. (glycogen content, 0·16%); magnification × 95; (*b*) Slice incubated with glucose (0·03M) for 20min. (glycogen content, 0·19%); magnification × 95. (*c*) Slice incubated with glucose (0·03M) for 45min. (glycogen content, 0·34%); magnification × 190. (*d*) Slice incubated with glucose (0·02M) for 45min. (glycogen content, 0·24%); magnification × 190. (*e*) Slice incubated with glucose (0·05M) for 45min. (glycogen content, 0·38%); magnification × 190. (*f*) Slice incubated with glucose (0·10M) for 45min. (glycogen content, 0·42%); magnification × 190.

the cells of the central part of the slice are able to synthesize glycogen. This is shown in Fig. 2(*b*). The slice has a thin part and a thick part. The thin part at the left is full of glycogen and the central zone of the thick part at the right has no glycogen. Slices full of glycogen were always obtained when liver slices less than 20 cells thick were incubated with glucose. It is noteworthy that the two or three first rows of cells at the periphery of the slice do not synthesize glycogen (Deane *et al.* 1947); this may be due to damage of the cells during slicing and incubation.

Figs. 2(*c*)–2(*f*) show the effect of oxygen pressure on the capacity of the cells of the slice to synthesize new glycogen. The depth of the rim of active cells that is shown in the histological section depends directly on oxygen pressure (Fig. 3). With an oxygen pressure of 2·7 atm. the central part of a slice about 35 cells thick becomes active (Fig. 2*g*). There is also a good correlation between the net synthesis of glycogen as measured by chemical method and the oxygen pressure (Fig. 4).

Figs. 5(*a*)–5(*c*) show histological sections of liver slices incubated with glucose and removed at different periods of time from 10 to 45 min. The depth of the rim reaches a maximum. Fig. 5(*a*), representing the section of a slice after 10 min. of incubation, has a rim about the same depth of a slice that was incubated for 45 min. (Fig. 5*c*). The intensity of the periodate–Schiff reaction, however, shows that with more prolonged incubation the slice has more glycogen, which agrees with chemical determinations. It has been suggested that the inability of the cells of the inner part of the slice to synthesize glycogen is due not only to lack of oxygen but also to a low concentration of glucose, which could be the result of a rather slow diffusion of the nutrient (Deane *et al.* 1947). To test this hypothesis, liver slices incubated with different concentrations of glucose were examined. The microscopic sections are shown in Figs. 5(*d*)–5(*f*). The depth of the rim containing newly synthesized glycogen is independent of glucose concentration. However, the intensity of the periodate–Schiff reaction increases with augmented concentration of glucose. The histochemical reactions confirm that the net glycogen synthesis measured by chemical methods is proportional to the concentration of glucose (Cahill *et al.* 1958; Niemeyer, 1955).

An important factor that influences glycogen synthesis by liver slices is the relative proportions of K^+ and Na^+ in the incubation medium (Hastings *et al.* 1952). We found that, in slices incubated with Na^+ instead of K^+, less glycogen was deposited in the active zone. In the Na^+-containing medium the depth of the active zone was slightly less than in the K^+-containing medium, confirming the data of Deane *et al.* (1947).

DISCUSSION

When liver slices prepared in a standard way and of such a thickness to permit optimum oxygen uptake are incubated in oxygen at 1 atm. pressure their inner cells cannot synthesize glycogen. On the other hand, all the cells from liver slices incubated at higher oxygen pressures can synthesize glycogen. The use of such higher oxygen pressures for the incubation of liver slices may bring several advantages. Let us consider a slice that is 35 cells thick (0·6 mm.) and is incubated at normal oxygen pressure. The wall of the tube of active cells, which forms a rim in the histological section of the slice, has a thickness of about ten cells. Only 57% of the cells of the slice are active if one neglects a few cells at the ends of the slice. By incubating the slice under an oxygen pressure higher than 1 atm., the relative proportion of active cells may be increased to 86%. Higher percentages of active cells cannot be obtained owing to damage to the two or three rows of peripheral cells. With a slice that is 20 cells thick and appears full of glycogen under 1 atm. oxygen pressure only, 75% of the cells of the slice are active owing to the fact that the damaged cells of the periphery represent a higher proportion of the total number of cells. These slices are rather thin, being about 0·35 mm. thick, and they are difficult to prepare and tear easily on agitation.

The behaviour of slices incubated under higher oxygen pressures probably gives a better indication of the synthetic activity of the tissue *in vivo*. This is not only due to a greater percentage of active cells, but also to the following consideration. In the slices incubated at normal pressure of oxygen, the interior cells not only do not synthesize glycogen but glycogen breakdown is also occurring. When liver slices of fed rats (high glycogen content) are incubated in a Krebs medium, the glycogen content of the tissue decreases during the incubation period (Niemeyer & Figueroa, 1956). This glycogen breakdown occurs in the inner cells of the slice and not apparently in the periphery. Therefore the net glycogen synthesis produced during the incubation of liver slices measures not the real capacity of the tissue to synthesize glycogen but rather the amount of glycogen synthesized by the peripheral cells minus the amount of glycogen broken down by the central cells.

The necessity of oxygen for glycogen synthesis arises from its dependence on ATP. Inhibition of ATP synthesis by 2,4-dinitrophenol inhibits glycogen synthesis in rat liver (Niemeyer, Figueroa & Gonzalez, 1956; Figueroa & Pfeifer, 1962). Disappearance of mitochondria in denervated muscle makes the tissue incapable of synthesizing glycogen (Carafoli, 1964). Histological examination of our liver slices incubated with glucose showed that the

zones that do not synthesize glycogen exhibit an almost complete disappearance of mitochondria but that they are conserved in the zones where glycogen is formed. These facts agree with those of Deane *et al.* (1947) and support the hypothesis that the necessity of oxygen for glycogen synthesis is dependent on mitochondria. The use of high oxygen pressures for the incubation of liver slices may be of value in biosynthesis studies on other substances that are dependent on ATP production.

The boundary between the active cells of the periphery and the inactive cells of the interior of the slice suggests that the cell needs a certain oxygen pressure to synthesize glycogen below which no synthesis occurs at all. According to Fuhrman & Field (1945), the maximum rate of oxygen consumption of rat-liver slices occurs with a slice thickness of 0·55mm., little change in oxygen uptake occurring in the thickness range 0·48–0·62mm. The maximal thickness that a tissue slice may attain without limitation of respiration of most interior cells through inadequate oxygen supply may be calculated by the equation (Warburg, 1928):

$$d' = \sqrt{8C_0 \cdot \frac{A}{D}} \qquad (1)$$

where C_0 is the oxygen concentration (in atmospheres) outside the slice, D the diffusion constant in the tissue and A the rate of oxygen uptake expressed in ml./min./ml. of tissue; d' represents the thickness of the tissue slice (cm.). Applying eqn. (1) to the oxygen uptake found by Fuhrman & Field (1945), namely 5×10^{-2}ml./min./ml., and a value of D equal to $1{\cdot}7 \times 10^{-5}$ml./min./cm.^{2}, a value of 0·059cm. is obtained for d', which agrees very well with the value determined experimentally. This agreement assures validity to the constant D for liver since its value is based on that obtained by Krogh (1919) with diaphragm. Field (1948) recommends 0·48–0·62mm. as the best thickness of rat-liver slices for studying respiration in an atmosphere of oxygen. The equation (Warburg, 1928):

$$C_1 = C_0 - \frac{A}{D} \cdot \frac{(d')^2}{8} \qquad (2)$$

gives the concentration of oxygen (C_1) in the centre of the tissue slice. The other letters have the same meaning as in eqn. (1). Applying eqn. (2), an oxygen pressure of zero at the centre of the slice is obtained with a slice thickness of 0·52mm. This agrees with the assumption that oxygen uptake is maximal in the cell with an oxygen pressure very close to zero. Eqn. (2) can also be used to calculate the oxygen pressure that exists in the boundary between cells that synthesize glycogen and cells that do not. By using the value of A found by Fuhrman & Field (1945) and the value of D applied by the same authors, this critical pressure varies between 0·09 and 0·17atm. This seems to be a limiting oxygen pressure, since under this pressure no glycogen is synthesized. This is in contrast with oxygen uptake, which is maximal with an oxygen pressure very close to zero. The difference is easily explained if we consider that glycogen synthesis depends on oxidative phosphorylation in the mitochondria. It could be that oxygen consumption in the mitochondria occurs at very low oxygen pressures whereas oxidative phosphorylation has a limiting oxygen pressure, the same as that found for glycogen synthesis. On the other hand, oxidative phosphorylation is a more fragile mechanism than oxygen consumption, as has been shown for isolated mitochondria (Lehninger, 1951). Therefore damage of mitochondria produced in the zones of very low oxygen pressure may become manifest only in the oxidative-phosphorylation mechanism.

It may be argued that oxygen may interfere in the process studied. Actually, oxygen at elevated pressure may have a harmful effect on the metabolism of mammalian tissue *in vitro* or in the activity of the enzymes. Oxygen uptake of rat-liver slices decreases with exposure to high oxygen pressure (Stadie, Riggs & Haugaard, 1945*b*). The same effect is observed in rat-brain slices or homogenates. The inhibition of brain metabolism by high oxygen pressure was similar with glucose, fructose, pyruvate or lactate as substrate (Stadie, Riggs & Haugaard, 1945*a*).

Many enzymes, especially those that depend on thiol groups for activity, are more or less inactivated by oxygen pressures at 1atm. or higher. Others are generally resistant (Haugaard, 1946). These effects on tissues or homogenates *in vitro* require oxygen pressures higher than those used in the present work, except for the toxic effect of oxygen on the enzyme systems that oxidize glucose or pyruvate, where 1atm. pressure is necessary (Haugaard, Hess & Itskovitz, 1957). On the other hand, the period of time required to harm the tissues or enzymes is always longer than 1hr. The incubation period we used was only 45min. Nevertheless, if there is some harmful effect of higher oxygen pressure on the mechanism of glycogen synthesis it is more than compensated for by the work of the increased number of active cells, since the net glycogen synthesis is greater with higher oxygen pressure.

The authors express their gratitude to Dr H. Niemeyer for encouraging the present study. This work was supported by Grant no. 59–35 from the 'Comisión de Ayuda a la Investigación Científica' financed by the Universidad de Chile and the Rockefeller Foundation in a joint programme.

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Biochem. J. (1966) **98**, 260

Metabolism of Saturated 1-^{14}C-Labelled Fatty Acids in the Silkworm *Bombyx mori* L.

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(*Received* 4 *May* 1965)

1. Only a small percentage of 1-^{14}C-labelled saturated fatty acids injected in the silkworm is respired as carbon dioxide. 2. The rate of utilization of fatty acids is low both at the larval and pupal stages. 3. The insect has the ability to elongate C_{12} and C_{16} saturated fatty acids and to desaturate C_{18} saturated fatty acids. 4. Much of the administered radioactivity is found in the triglyceride fraction, followed by the phospholipid and diglyceride fractions. 5. Diglycerides seem to be the transport form of fatty acids. 6. The insect seems to metabolize both natural and unnatural fatty acids in the same manner.

Although earlier respiratory quotient measurements implicated fat as the energy source in several insect species (Weis-Fogh, 1952), our studies indicate that this may not be so in the silkworm (Sridhara, 1965; Sridhara & Bhat, 1965*c*). Chino & Gilbert (1964), working with *Hyalophora* silkmoths, showed that injected palmitic acid is carried mainly as diglycerides attached to a protein, whereas newly synthesized fatty acids from [1-^{14}C]acetate were found in the monoglyceride fraction of silkworm pupal lipids (Sridhara & Bhat, 1965*a*). These raised questions as to whether: (i) the silkworm metabolizes preformed and derived fatty acids separately; (ii) differences exist between larval and pupal stages in fatty acid metabolism; (iii) fatty acids are utilized as energy source during metamorphosis and pupal development. The present paper describes experiments designed to answer these questions by studying the fate of saturated 1-^{14}C-labelled fatty acids.

MATERIALS AND METHODS

Treatment and extraction of insects. Mature silkworm larvae of *Bombyx mori* L. weighing 1·1–1·2g. and 5-day-old pupae weighing 0·5–0·6g. were used. Larvae and pupae of approximately the same weight were used in each experiment, but weights differed slightly from experiment to experiment. The methods adopted for injection, lipid extraction and fractionation, fatty acid analysis and isolation of different organs have been detailed by Sridhara & Bhat (1964, 1965*a*). For the intestine the whole gut was used and integument was free of adhering muscle. [1-^{14}C]-Palmitic acid (sp. activity 145μc/mg.), -stearic acid (sp. activity 43·3μc/mg.) and -lauric acid (sp. activity 13μc/mg.) were obtained from The Radiochemical Centre, Amersham, Bucks. Stock solutions of these fatty acids were prepared in ethanol. Just before use, ethanol was evaporated and the fatty acid was taken up as a dispersion in Tween 80, which was used as a 2% suspension in phosphate buffer, pH 7·4.

Estimation of expired $^{14}CO_2$. Larvae 3 or 4 days after the fourth moult and weighing 0·5–0·6g. were injected with 0·5μc of the fatty acid, and were allowed to feed on mulberry leaves for 1hr. and then transferred to separate Warburg flasks (15ml.). The CO_2 expired was absorbed into 20% (w/v) KOH contained in the centre well. After the times indicated in the Tables, commencing from the removal of larvae from the mulberry leaves, the alkali was transferred to centrifuge tubes by repeated washing and the carbonate was precipitated with $BaCl_2$ and allowed to stand overnight. The precipitate was centrifuged down, washed twice with water and twice with ethanol, dried, weighed and plated for counting.

RESULTS

Tables 1, 2 and 3 show the results of experiments carried out with lauric acid, palmitic acid and stearic acid respectively. Subsequent pupation of the larvae after the injection of radioactive fatty acid is indicated in the first column of each Table and is represented as ∞. Carbon dioxide could be collected only for 8hr. since the larvae became weak and the respiration rate decreased considerably thereafter. However, such larvae, if allowed to feed, grew just as well as the normal ones.

The results show that the amount of radioactivity released as carbon dioxide is low, and this compares favourably with the diminution of radioactivity recoverable from the lipid. The absence of absolute correspondence is due to the use of different larvae, of different ages, and different timings. The

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Table 1. *Incorporation of* [1-^{14}C]*lauric acid into the lipids of the silkworm*

Experimental details are given in the text.

Time after injection (hr.)	Percentages of radioactivity recovered in lipid	Percentages of radioactivity in respired CO_2	Percentage of radioactivity in the saponifiable fraction				Percentage of radioactivity in lipid fractions eluted			
			Palmitic acid	Stearic acid	Oleic acid	Lauric acid	Eluent..... 6% Ether Fraction...Triglycerides and free fatty acids	25% Ether Diglycerides	100% Ether Monoglycerides	Methanol Phospholipids
Larva										
1	90	5	4·0	2·0	3	84	61·6	7·4	6·4	12·9
2	—	7	—	—	3	—	—	—	—	—
4	89	11	8·7	5·8	3	77	62·3	6·3	4·1	13·0
8	85	15	11·0	7·0	3	70	64·1	4·9	3·9	13·0
12	79	—	17·4	9·2	3	65	66·8	3·9	3·4	13·2
24	70	—	21·0	11·4	3	59	69·2	3·2	3·3	13·3
48	62	—	24·5	13·6	3	50	71·4	2·0	2·0	13·3
∞	51	—	19·2	17·3	16	41	69·6	2·6	2·0	16·5
Pupa										
1	95	—	4–5	1–2	0–1	80–85	46·3	9·8	6·1	12·4
4	91	—	4–5	1–2	0–1	80–85	53·6	7·4	3·3	12·8
8	88	—	4–5	1–2	0–1	80–85	54·6	6·4	3·0	13·2
12	85	—	4–5	1–2	0–1	80–85	55·2	5·3	3·4	13·2
24	83	—	4–5	1–2	0–1	80–85	58·9	5·1	3·0	13·4
48	81	—	4–5	1–2	0–1	80–85	62·8	4·1	3·3	13·5
120	74	—	4–5	1–2	0–1	80–85	68·0	3·3	4·0	12·6

Table 2. *Incorporation of* [1-^{14}C]*palmitic acid into the lipids of the silkworm*

Experimental details are given in the text.

Time after injection (hr.)	Percentage of radio-activity recovered in lipid	Percentage of radio-activity in respired CO_2	Percentage of radioactivity in the saponifiable fraction			Percentage of radioactivity in lipid fractions eluted			
			Palmitic acid	Stearic acid	Oleic acid	Eluent... Fraction... 6% Ether Triglycerides and free fatty acids	25% Ether Diglycerides	100% Ether Monoglycerides	Methanol Phospholipids
Larva									
1	93·0	3	86	5	1	55·0	14·5	2·1	13·9
2	90·6	7	—	—	—	—	—	—	—
4	88·5	10	83	8	3	58·0	12·3	3·0	14·1
8	84·4	12	80	10	4	60·0	9·0	3·0	14·1
12	81·5	—	76	13	5	62·1	7·5	4·0	15·3
24	78·6	—	71	15	7	64·1	6·8	3·5	15·0
48	73·4	—	67	17	9	59·8	7·9	3·9	15·2
∞	70·5	—	43	24	26	66·3	6·1	2·0	17·6
Pupa									
1	94·0	—	86	3	1	34·0	33·7	3·0	15·8
4	91·0	—	82	5	3	41·9	27·2	4·3	18·8
8	88·0	—	80	6	4	50·2	21·5	5·1	20·5
12	85·0	—	77	7	5	53·4	19·6	5·2	21·2
24	83·5	—	75	9	6	55·0	16·0	6·0	23·3
48	81·0	—	73	11	7	56·0	12·0	6·0	24·2
120	77·0	—	71	13	7	59·0	8·8	5·6	24·0

Table 3. *Incorporation of* [1-^{14}C]*stearic acid into the lipids of the silkworm*

Experimental details are given in the text.

Time after injection (hr.)	Percentage of radio-activity recovered in lipid	Percentage of radio-activity in respired CO_2	Percentage of radioactivity in saponifiable fraction			Percentage of radioactivity in lipid fractions eluted			
			Palmitic acid	Stearic acid	Oleic acid	Eluent..... 6% Ether Fraction...Triglycerides and free fatty acids	25% Ether Diglycerides	100% Ether Monoglycerides	Methanol Phospholipids
Larva									
1	91	3	0·5	88·0	3·4	44	24	3	21
2	—	4	—	—	—	—	—	—	—
4	89	6	2·3	84·0	7·1	46	21	4	21
8	87	10	3·1	81·0	10·8	46	18	3	25
12	80	—	4·0	78·5	12·3	47	16	3	27
24	75	—	4·5	75·0	14·8	47	14	4	27
48	73	—	5·0	71·0	17·1	50	12	3	27
∞	68	—	10·2	52·1	31·8	52	5	2	32
Pupa									
1	92	—	0·0	93·0	2·1	39	24	11	20
4	90	—	1·0	90·1	3·6	37	22	10	21
8	89	—	1·2	87·5	6·3	40	20	9	22
12	85	—	1·2	84·3	8·2	43	17	7	22
24	80	—	1·6	81·0	10·1	46	15	6	21
48	79	—	2·0	79·2	12·4	50	13	3	—
120	75	—	2·2	77·5	13·8	54	11	—	23

amount of radioactivity lost during the pupal stage is much lower than that during the larval stage. In all cases 50–60% of the administered radioactivity is found in the lipid fraction even after 8 days of administration at the larval stage, and about 70% is recovered at the pupal stage after 5 days.

The amounts of radioactivity found in the various fatty acids show that the rate of conversion into other fatty acids depends on the fatty acid used. Stearic acid is converted to a greater extent into oleic than into palmitic acid, conversion of palmitic acid into stearic acid is much greater than that into oleic acid, and lauric acid is converted mainly into palmitic acid and stearic acid. However, in all cases the rate of conversion at the pupal stage is much lower than at the larval stage.

The percentage of radioactivity that is eluted in the various fractions is shown in the respective Tables. The fractions eluted with light petroleum, 1% (v/v) ether in light petroleum and 10% (v/v) ether in light petroleum respectively have not been included, since in all the cases the percentage of radioactivity eluted was equal to or less than 1·0. Within 1hr. after injection most of the radioactivity in the lipid is distributed among the triglyceride, diglyceride and phospholipid fractions. The amount of radioactivity recovered in the diglycerides was small with [1-^{14}C]lauric acid, higher with [1-^{14}C]-stearic acid and highest with [1-^{14}C]palmitic acid. The percentage of radioactivity found in the diglyceride fraction is slightly higher at the pupal stage. The percentage of radioactivity found in the phospholipid fraction at both stages is somewhat higher with [1-^{14}C]stearic acid than with the other two labelled acids. The high radioactivity found in the diglyceride fraction at the beginning decreases slowly with time, whereas that of the triglyceride fraction increases.

Table 4 shows the distribution of the radioactivity among different organs of the silkworm 8hr. and 24hr. after the administration of the fatty acid. The pattern of distribution appears to be similar for all fatty acids in that the fat body carries the maximum activity, followed by integuments, silk glands and intestines.

DISCUSSION

The retention of a considerable amount of injected radioactivity in the lipid and expiration of only small quantity thereof in carbon dioxide indicates a low utilization of the labelled fatty acid by the insect. This is further supported by the negligible utilization of the injected fatty acid both during metamorphosis and pupal development. The decrease in the amount of ^{14}C recovered in carbon dioxide might be due to the dilution of the substrate or conversion of the fatty acid into a non-utilizable form.

In the rat, which is known to utilize fat as the energy source, on the other hand, about 50% of [1-^{14}C]palmitic acid, -trilaurin and -tripalmitin appears as carbon dioxide within 2hr. and as much as 70% within 3·5hr. (Geyer, Chipman & Stare, 1948; Lerner, Chaikoff, Entenmann & Dauben, 1949; McCalla, Gates & Gordon, 1957). This finding, when considered together with the previous observation of the inability of silkworm intestinal mitochondria to oxidize fatty acids (Sridhara, 1965) on the one hand and the non-interconversion of fat and carbohydrate in this insect (Sridhara & Bhat, 1965c) on the other, is indicative of the unsuitability of fat as a source of energy for this insect at least at the stages studied. Domrose & Gilbert (1964) have shown that fat is utilized by males of *H. cecropia* only as adults, whereas females use fat both during adult development and adult life.

The fact that the radioactivity of the administered fatty acid is found in other fatty acids also shows that the silkworm is capable not only of elongating saturated fatty acids but also of desaturating stearic acid to oleic acid. The recovery of radioactivity from [1-^{14}C]stearic acid in palmitic acid as well as that of radioactivity from [1-^{14}C]-lauric acid in palmitic acid may be due to the fact that the ^{14}C of [1-^{14}C]stearic acid enters the C_2

Table 4. *Incorporation of* [1-^{14}C]*-stearic acid, -palmitic acid and -lauric acid into the lipids of the tissues of the silkworm*

	Percentage distribution of radioactivity					
	[1-^{14}C]Stearic acid		[1-^{14}C]Palmitic acid		[1-^{14}C]Lauric acid	
Tissue	After 8hr.	After 24hr.	After 8hr.	After 24hr.	After 8hr.	After 24hr.
Intestines	9·5	11·3	10·5	9·8	7·5	6·8
Silk glands	6·3	7·3	10·6	11·4	7·6	7·8
Fat body	47·2	46·1	52·8	54·5	61·5	61·4
Integuments	23·6	25·2	16·8	17·2	16·5	16·7

pool and is used for the new synthesis of these fatty acids. The observation that more radioactivity passes into oleic acid during metamorphosis is in conformity with that made previously with [1-^{14}C]acetate and with respect to the increase in oleic acid during metamorphosis at the expense of saturated fatty acids (Sridhara & Bhat, 1965*b*). Another conclusion that emerges from these results is that the silkworm metabolizes an unnatural fatty acid (lauric acid is not present in the silkworm) in a manner identical with that used for the natural acids, and that pupae metabolize fatty acids more slowly than do larvae.

The recovery of a high percentage of radioactivity in the diglyceride fraction released from the fat body under the influence of some factor in the haemolymph (Teitz, 1962; Chino & Gilbert, 1965*a*) is not surprising in view of the fact that diglycerides are the transport form of lipids in the three insects studied. In the silkworm, if one considers the specific activity of the diglyceride fraction after injection with [1-^{14}C]-palmitic acid and -stearic acid, it is always equal to or higher than that of triglycerides formed during the first 2hr. This means that the fat body endergonically carries out the synthesis of triglycerides via diglycerides and releases fat from fat body as diglycerides, and this may explain the low rate of utilization of fatty acids. In mammals it has been definitely shown that plasma unesterified fatty acid is the transport form of lipid available for tissues as substrate for oxidation, and that when its concentration decreases fatty acid oxidation is inhibited (Lossow & Chaikoff, 1955; McCalla *et al.* 1957; Fredrickson & Gordon, 1958; Steinberg, 1963). If this theory holds good for insects also (unesterified fatty acid has been found in many insects including the silkworm; Sridhara & Bhat, 1965*b*), then it may be postulated that the fatty acid administered is immediately converted into diglycerides, triglycerides and phospholipids, thus obviating the need for its existence as unesterified fatty acid in blood. The hypothesis would also explain the enhanced radioactivity of the carbon dioxide released at the beginning of the experiment. In fact, Chino & Gilbert (1965*b*) have shown that fatty acids are esterified to diglycerides and triglycerides at a fast rate in the fat body of *H. cecropia* silkmoths.

It is only in the distribution of radioactivity among various fractions that some differences are seen between preformed and derived fatty acids. The radioactivity of fatty acids synthesized from [1-^{14}C]acetate is greater in the monoglyceride fraction at the pupal stage; other differences were also observed between the larval and pupal stages (Sridhara & Bhat, 1965*a*). But no such differences are observed with the labelled fatty acids. This may be either due to the fact that the insect has to deal with all the different fatty acids synthesized from [1-^{14}C]acetate in contradistinction to only one fatty acid administered in the present work, or due to the differences in the mode of transport itself. However, the pattern of distribution of the radioactivity among different tissues is not inconsistent with that observed previously in connexion with the metabolism of [1-^{14}C]acetate (Sridhara & Bhat, 1965*a*).

This work was supported by grants from U.S. Public Law 480 funds. Our thanks are due to Professor P. S. Sarma for the use of counters.

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The Metabolism of [2-^{14}C]Indole in the Rat

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(*Received* 22 *June* 1965)

1. [2-^{14}C]Indole has been synthesized from [^{14}C]formate and *o*-toluidine via *N*[^{14}C]-formyltoluidine. 2. When fed to rats, the ^{14}C of [^{14}C]indole (dose 70–80mg./kg. body wt.) is fairly rapidly excreted, and in 2 days an average of 81% appears in the urine, 11% in the faeces and 2·4% as carbon dioxide in the expired air. 3. Radioactivity is excreted in the urine as indoxyl sulphate (50% of the dose), indoxyl glucuronide (11%), oxindole (1·4%), isatin (5·8%), 5-hydroxyoxindole conjugates (3·1%), *N*-formylanthranilic acid (0·5%) and unchanged indole (0·07%). The faeces contain indoxyl sulphate (0·4% of the dose) and indole (0·2%), but the major metabolites have not been identified. 4. Fed to rats with biliary cannulae an average of 5·6% of a dose of [^{14}C]indole (20–60mg./kg. body wt.) is excreted in the bile in 2 days. Radioactivity is present as indoxyl sulphate (0·8% dose) and 5-hydroxyoxindole conjugates (0·6%). 5. Rats further metabolize indoxyl into *N*-formylanthranilic acid and anthranilic acid, and oxindole into 5-hydroxyoxindole. 6. With rat-liver microsomes plus supernatant under aerobic conditions, indole gives indoxyl, oxindole, possibly isatin, *N*-formylanthranilic acid and anthranilic acid, but under anaerobic conditions gives only oxindole. Similarly, under aerobic conditions, oxindole gives 5-hydroxyoxindole, anthranilic acid and *o*-aminophenylacetic acid. 7. Indole is metabolized by two pathways, one via indoxyl to isatin, *N*-formylanthranilic acid and anthranilic acid, and the other via oxindole to 5-hydroxyoxindole and possibly to *o*-aminophenylacetic and anthranilic acid. 8. The following new compounds are described: 4-*hydroxy*-2-*nitrophenylacetic acid*, 3-, 4- and 5-*benzyloxy*-2-*nitrophenylacetic acid*, 5- and 7-*hydroxyoxindole* and 5-*aminoacridine indoxyl sulphate*.

The indole nucleus is a fundamental structure in a number of biologically important compounds, but the metabolic fate of indole itself has not been fully elucidated. It has been known for a considerable time that indole is converted into 3-hydroxyindole (indoxyl) in the animal body (Baumann, 1877; Mester, 1888) and this has been repeatedly confirmed (see Williams, 1959). The indoxyl formed is excreted in the urine primarily as an ethereal sulphate (urinary indican) and to a smaller extent as a glucuronide. Preliminary studies from this Laboratory (Kaighen, 1961) had shown that indoxyl was not the only metabolite of indole in the rabbit, and evidence was obtained which suggested that indole was hydroxylated to a small extent in the 5-position. Further, it was reported by Terashima (1957) that anthranilic acid was a metabolite of indole in the rabbit.

By using [2-^{14}C]indole in the rat, we have shown that, although indoxyl is, in fact, the major metabolite of indole, there are other metabolites in urine, and these have been identified as oxindole, isatin, *N*-formylanthranilic acid, anthranilic acid and the ethereal sulphate and glucuronide of 5-hydroxyoxindole. There is also a small output of $^{14}CO_2$ in the expired air of rats receiving [^{14}C]indole and this appears to be derived from indole via indoxyl and *N*-formylanthranilic acid.

EXPERIMENTAL

Materials

Synthesis of [2-^{14}C]*indole*. The synthesis was based on the pyrolysis of *N*-formyl-*o*-toluidine in the presence of potassium *tert.*-butoxide (Tyson, 1955). Sodium [^{14}C]formate (11·1mg., 1mc), 99% (w/v) formic acid (0·65ml.) and 85% (w/v) H_3PO_4 (0·011ml.) were heated together in a 5ml. flask and the mixture was distilled. The distillate of [^{14}C]-formic acid (0·73g., 0·57mc) was collected in a 25ml. flask cooled in acetone–solid CO_2; *o*-toluidine (1·8g.) was added and the mixture was heated under reflux on a water bath for 3hr. After cooling, the mixture was seeded with a crystal of *N*-formyl-*o*-toluidine and left overnight to crystallize. The solid product was filtered, washed with 0·1N-HCl (10ml.) and then water (10ml.), dried *in vacuo* over P_2O_5 and twice recrystallized from benzene–light petroleum (b.p. 40–60°) (1:2, v/v) to give *N*[^{14}C]-formyl-*o*-toluidine, m.p. 61°

(1·57g., 0·62mc). This was added to a freshly prepared solution of potassium *tert.*-butoxide (2·0g.) in 2-methylpropan-2-ol (50ml.) in a 100ml. flask and the mixture distilled to remove the solvent. The solid residue was then heated in a metal bath, the temperature of which was slowly raised to 250°, when the residue melted and excess of potassium *tert.*-butoxide distilled over. This slow heating prevented decrepitation of the material and increased the final yield of indole. The temperature of the metal bath was then raised to 350–360° for 20min., after which the flask was removed from the bath and the contents were cooled in a stream of N_2. The pyrolysis product was decomposed by treatment with 0·1N-HCl (10ml.) and the mixture was then extracted with four 100ml. portions of ether. The combined ether extracts were concentrated *in vacuo* at room temperature to about 3ml., which was then chromatographed on a column (1cm. diam.) of silica gel (Florisil, 50g.). The column was developed with chloroform–light petroleum (b.p. 40–60°) (1:2, v/v) and the first 600ml. of eluate was collected and evaporated to dryness *in vacuo* to give crude [2-^{14}C]indole (0·445g.). This was further purified by chromatographing similarly on a second column of silica gel. Recrystallization from light petroleum (b.p. 40–60°) containing a little ether gave [2-^{14}C]indole as white plates, m.p. 53° (0·174g., 97μc, equivalent to 19·4% of the theoretical radiochemical yield).

Further elution of the silica-gel columns gave unchanged *N*-formyl-*o*-toluidine, which was recovered and converted into indole by pyrolysis as before. Additional radioactive indole was also obtained by entrainment of radioactive residues in repeated syntheses starting with unlabelled formic acid. In this way two further batches of [2-^{14}C]indole (total of 1·43g. and 66μc) and two batches of *N*[^{14}C]-formyl-*o*-toluidine (total of 3·47g. and 92μc) were recovered. The remainder of the original radioactivity (about 50% of the theoretical radiochemical yield) was accounted for as polymerized end products.

The [2-^{14}C]indole was chromatographed on paper in the solvent systems described under 'Chromatography'. Development of the chromatograms with the different detecting reagents and determination of the radioactivity by an end-window Geiger–Müller counter tube scanner revealed no impurities and showed that radioactivity was associated only with the indole.

6-*Hydroxyoxindole*. This compound, m.p. 243° (decomp.), has been obtained previously from 4-methoxy-2-nitrotoluene by the Reissert synthesis (Wieland & Unger, 1963) but the yields were poor. Better yields were obtained by catalytic reduction of 4-hydroxy-2-nitrophenylacetic acid. 4-Amino-2-nitrophenylacetic acid (Gabriel & Meyer, 1881) (1g.) in 8N-H_2SO_4 (30ml.) was cooled to 0° and treated with $NaNO_2$ (0·4g. in 2ml. of water) with stirring. The diazotized mixture was left for 1hr. at 0° and then added slowly to 150ml. of 25N-H_2SO_4 at 110° and kept at this temperature for 5min. The solution was cooled rapidly, diluted to 250ml. with water and extracted with ether (3×200ml.). The combined extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness *in vacuo* to an orange semi-crystalline residue, which was dissolved in hot ethyl acetate (charcoal) and reprecipitated by the dropwise addition of light petroleum (b.p. 60–80°). After leaving overnight the 4-*hydroxy-2-nitrophenylacetic acid* was filtered off, dried and recrystallized from benzene–light petroleum (b.p. 60–80°) to give pale-yellow needles (0·1–0·5g.), m.p. 180° (decomp.) (Found: C, 48·6; H, 3·9; N, 6·6. $C_8H_7NO_5$ requires C, 48·7; H, 3·6; N, 7·1%). The acid (1g.) in acetic acid (40ml.) was hydrogenated at 40lb./in.^{2} for 2hr. with 10% palladium–charcoal (0·1g.) as catalyst. The catalyst was filtered off and the solvent removed to leave a white crystalline residue, which was recrystallized from hot water (charcoal). 6-Hydroxyoxindole was filtered off, washed with water and dried to give white needles (0·35g.), m.p. 265° (decomp.) (Found: C, 64·0; H, 5·0; N, 9·4. Calc. for $C_8H_7NO_2$: C, 64·4; H, 4·7; N, 9·4%).

Better yields of 6-hydroxyoxindole were obtained by catalytic reduction of 4-benzyloxy-2-nitrophenylacetic acid. A solution of 1·5g. of 4-benzyloxy-2-nitrophenylpyruvic acid (Stoll, Troxler, Peyer & Hofmann, 1955) in 15ml. of 0·05N-NaOH was treated dropwise at 0° with H_2O_2 (6%, w/v) until the solution no longer gave a red colour with excess of NaOH. The resulting solution was then acidified with 2N-HCl and the precipitate that formed was filtered off, washed with water, dried and recrystallized from benzene to give pale-yellow needles (1·25g.) of 4-*benzyloxy-2-nitrophenylacetic acid*, m.p. 157° (Found: C, 62·9; H, 4·6; N, 5·0. $C_{15}H_{13}NO_5$ requires C, 62·7; H, 4·6; N, 4·9%). This material (2·0g.) was then converted into 6-hydroxyoxindole by catalytic reduction as described above. The 6-hydroxyoxindole (yield, 0·5g.) prepared by this method had m.p. 265° (decomp.) and was shown by thin-layer chromatography on alumina to be identical with that prepared by reduction of 4-hydroxy-2-nitrophenylacetic acid.

5-*Hydroxyoxindole*. This compound was prepared similarly by the reduction of 5-benzyloxy-2-nitrophenylacetic acid. A solution of 5-benzyloxy-2-nitrophenylpyruvic acid (Stoll *et al.* 1955) (8g. in 80ml. of 0·05N-NaOH) was treated at 0° with H_2O_2 (6%, w/v). The solution was acidified with 2N-HCl and the precipitate was filtered off and recrystallized from benzene to give colourless needles (6·8g.) of *5-benzyloxy-2-nitrophenylacetic acid*, m.p. 168° (Found: C, 62·9; H, 4·7; N, 4·9. $C_{15}H_{13}NO_5$ requires C, 62·7; H, 4·6; N, 4·9%). This material (6·7g.) was then hydrogenated in the presence of 10% palladium–charcoal catalyst to give *5-hydroxyoxindole* (1·75g.), m.p. 269° (decomp.), which formed colourless needles from water (Found: C, 64·4; H, 4·7; N, 9·5. $C_8H_7NO_2$ requires C, 64·4; H, 4·7; N, 9·4%).

7-*Hydroxyoxindole*. This compound was prepared similarly by the reduction of 3-benzyloxy-2-nitrophenylacetic acid. 3-Benzyloxy-2-nitrophenylpyruvic acid (Beer, Clarke, Khorana & Robertson, 1948) (4g. in 50ml. of 0·05N-NaOH) was treated at 0° with H_2O_2 as above to give colourless needles (3·1g.) of *3-benzyloxy-2-nitrophenylacetic acid*, m.p. 161°, after recrystallization from benzene (Found: C, 62·25; H, 5·1; N, 5·0. $C_{15}H_{13}NO_5$ requires C, 62·7; H, 4·6; N, 4·9%). This (1·2g.) was then hydrogenated in the presence of 10% palladium–charcoal catalyst to give *7-hydroxyoxindole* as colourless needles (0·4g.) (m.p. 251°, decomp.) from water (Found: C, 64·3; H, 4·8; N, 9·6. $C_8H_7NO_2$ requires C, 64·4; H, 4·7; N, 9·4%).

5- *and* 6-*Hydroxyindole*. These were prepared as required by reductive debenzylation of the corresponding benzyloxyindoles. 5-Benzyloxyindole, m.p. 102°, or 6-benzyloxyindole, m.p. 116° (50mg.; Koch–Light Laboratories Ltd., Colnbrook, Bucks.), in 30ml. of methanol was hydrogenated at 40lb./in.^{2} for 2hr. with 0·01g. of 10% palladium–charcoal as catalyst. The catalyst was filtered off and the solvent

removed *in vacuo* at 25°. The colourless solid residues of 5-hydroxyindole, m.p. 116° [Stoll *et al.* (1955) give m.p. 108°], and 6-hydroxyindole, m.p. 125°, were used without further purification since they became oxidized and darkened rapidly on exposure to air.

5-Aminoacridine indoxyl sulphate. This was prepared by treating a solution of potassium indoxyl sulphate (0·50g.) in water (25ml.) with a solution of 5-aminoacridine hydrochloride (0·46g.) in hot water (20ml.). The salt, which separated immediately, was filtered off, washed with cold water and recrystallized from water. *5-Aminoacridine indoxyl sulphate* formed yellow crystals (0·6g.), m.p. 215° (decomp.) [Found, SO_4, 23·3; $C_{13}H_{10}N_2$ (5-aminoacridine), 48·2%. $C_{21}H_{17}N_3O_4S$ requires SO_4, 23·5; $C_{13}H_{10}N_2$, 47·7%].

Other materials. Potassium indoxyl sulphate (Boyland, Sims & Williams, 1956), *N*-formylanthranilic acid, m.p. 168° (Meyer & Bellmann, 1886), oxindole, m.p. 126° (Di Carlo, 1944), indirubin (sublimes at 244°) (Forrer, 1884), indigotin (Thomas, Bloxam & Perkin, 1909) and isatan (Hansen, 1924) were synthesized and purified. Indole, m.p. 54°, and isatin, m.p. 201°, were purchased (Koch–Light Laboratories Ltd.) and purified.

Chromatography

Samples of urines (0·2ml.) and of concentrates (0·02ml.) prepared from the urines by adsorption on to partially deactivated charcoal (Asatoor & Dalgliesh, 1956) were chromatographed on sheets of Whatman no. 1 paper, 23cm. square, in solvent *A* (propan-2-ol–aq. NH_3 (sp.gr. 0·88)–water; 8:1:1, by vol.), and then at right-angles in solvent *B* (butan-1-ol–acetic acid–water; 4:1:1, by vol.). Paper chromatograms were also prepared in solvents *C* (benzene–propionic acid–water; 20:14:1, by vol.), *D* (butan-1-ol–pyridine–water; 1:1:1, by vol.), *E* (phenol–aq. NH_3 (sp.gr. 0·88)–water; 160:1:40, by vol.) and *F* (aq. 20% KCl). Radioautographs were taken from the paper chromatograms, which were also examined in u.v. light (254mμ; Hanovia Chromatolite lamp), and developed by spraying with the following reagents: 2% (w/v) *p*-dimethylaminobenzaldehyde in 2N-HCl (Ehrlich's reagent); 0·05% (w/v) 2,6-dichloroquinonechloroimide in ethanol followed by aq. saturated $NaHCO_3$ (Gibbs's reagent). Radioactive spots on radioautographs were identified by the R_F values and colour reactions of corresponding spots on paper chromatograms (see Table 1).

5- and 6-*Hydroxyoxindole* were separated on thin-layer chromatoplates (De Saga) of alumina (E. Merck A.-G., Darmstadt, Germany) developed in solvent *G* (*n*-hexane–propanol-1-ol; 10:1, v/v) (see Table 2).

Animal experiments

Collection of excreta. Female albino rats [Wistar strain, weight about 200g.; Animal Supplies (London) Ltd.] were maintained on 25g./day of Diet 41B (Joseph Rank Ltd.), with free access to water. [2-^{14}C]Indole and unlabelled indole were administered orally as solutions in arachis oil (5%, w/v). Oxindole, 5- and 6-hydroxyoxindole, indoxyl acetate, indoxyl sulphate, *N*-formylanthranilic acid and

Table 1. *Chromatography and colour reactions of possible metabolites of indole*

Ascending chromatograms were run on square sheets of Whatman no. 1 paper in solvent *A* and then at right-angles in solvent *B* (see the text). Metabolites were detected by spraying with Ehrlich's reagent or Gibbs's reagent followed by aq. saturated $NaHCO_3$, or by the fluorindal reaction, i.e. Ehrlich's reagent followed by aq. saturated sodium acetate solution, with which indoxyl derivatives give a red colour. Fluorescence was observed in u.v. light (254mμ; Hanovia Chromatolite lamp).

	R_F in		Colour with			
Metabolite	Solvent *A*	then solvent *B*	Ehrlich's reagent	Gibbs's reagent	Fluorindal reaction	Fluorescence
Indole	0·94	0·95	Purple	Purple	–	None
Indoxyl sulphate	0·52	0·44	Brown	Purple	+	Blue
Indoxyl glucuronide	0·21	0·42	Purple→brown	Purple	+	Purple
5-Hydroxyindole	0·85	0·85	Purple	Purple	–	
6-Hydroxyindole	0·73	0·87	Blue	Purple	–	
Oxindole	0·79	0·81	Orange*	Red	–	q†
5-Hydroxyoxindole sulphate	0·35	0·21	Orange*	Red	–	q†
5-Hydroxyoxindole glucuronide	0·07	0·20	Orange*	Red	–	q†
5-Hydroxyoxindole	0·58	0·65	Orange*	Purple–red	–	q†
6-Hydroxyoxindole	0·58	0·68	Orange*	Purple–red	–	q†
7-Hydroxyoxindole	0·85	0·80	Orange*	Blue	–	q†
Indirubin	0·63	0·81	—	—	–	q†
Indigotin	0·60	0·75	—	—	–	q†
Isatin	0·66	0·81	—	—	–	q†
N-Formylanthranilic acid	0·52	0·87	Yellow	Blue–green	–	Blue
Anthranilic acid	0·41	0·87	Yellow	Blue–green	–	Blue

* Colour developed after 48hr.

† q means quenching of background fluorescence.

Table 2. *Thin-layer chromatography of some hydroxyoxindoles and bis-indoles*

Alumina chromatoplates were used. For solvent *G*, Ehrlich's reagent and Gibbs's reagent see the text. DSA is 1% (w/v) diazotized sulphanilic acid to which an equal volume of 2 N-Na_2CO_3 was added just before spraying.

Compound	R_F values in Solvent *G*	R_F values in $CHCl_3$	Colour with Ehrlich's reagent	Colour with Gibbs's reagent	Colour with DSA
5-Hydroxyoxindole	0·61		Orange	Greyish violet	Orange
6-Hydroxyoxindole	0·35				
7-Hydroxyoxindole	0·07				
Hydroxyoxindole fraction from indole urine	0·61				
Hydroxylation products of oxindole by ascorbic acid–iron system*	0·61				
	0·30				
Isatin†	0·21	0·22	—	Red	—
Isatan†	0·38	0·35	—	—	—
Indirubin†	0·40	0·45	—	—	—
Oxindole	0·52	0·50	—	Red	—

* Oxindole (240 mg.) in phosphate buffer, pH 6·7 (250 ml.), containing EDTA (disodium salt) (7·6 g.), ascorbic acid (3 g.) and $FeSO_4$ (0·2 g.) was shaken with O_2 for 6 hr. (see Udenfriend *et al.* 1954). The solution was extracted with ether (3 × 100 ml.). The extract was dried (anhydrous Na_2SO_4) and evaporated to dryness, and the excess of oxindole removed from the residue with benzene (25 ml.). The residue was dissolved in a little methanol for chromatography.

† These compounds are coloured.

anthranilic acid were administered as aqueous suspensions; 5- and 6-hydroxyindole were administered as 5% solutions in propylene glycol.

Rats given [2-^{14}C]indole were placed in a plastic metabolism chamber through which a current of air was drawn. The outgoing air was passed through two wash-bottles in series containing acetone at −30° to trap volatile organic compounds and then through two wash-bottles containing 4 N-NaOH to remove respiratory CO_2. Urine and faeces were collected daily. After 3 days the animals were killed and the radioactivity of the tissues was determined. The urine was examined for metabolites qualitatively by chromatography and radioautography, and quantitatively by reverse isotope dilution.

Collection of bile. Polythene cannulae were fitted into the common bile duct of rats and the animals were then allowed to recover from the operation for 24 hr. (Stewart & Harrison, 1961). They were then dosed orally with [2-^{14}C]-indole and bile was collected for 48 hr. into a tube kept at −10° to minimize any possible action of biliary enzymes on conjugates. The total radioactivity of the bile was estimated as described below and that of any metabolite present by reverse isotope dilution.

Determination of radioactivity. Radioactive materials were counted as samples of infinite thickness with high-sensitivity low-background counting equipment (Panax Equipment Ltd.) incorporating the MX 157 anti-coincidence Geiger Counter assembly (Mullard Ltd.). Urine and bile were counted as such, tissues and faeces as homogenates in water, and the $^{14}CO_2$ in the expired air as $Ba^{14}CO_3$. Samples were counted for periods of time sufficient to record at least 10^3 counts net and to obtain a standard error of the mean less than 2%.

Qualitative identification of metabolites

A portion (5 ml. containing about 1·5 μc) of the 24 hr. urine from a rat dosed orally with [2-^{14}C]indole (15·75 mg., 9·0 μc) was diluted to 20 ml. with water acidified to pH 3 with acetic acid, and then shaken with 1 g. of charcoal that had been partially deactivated with *n*-octadecylamine. The charcoal was filtered off and then washed with water (3 × 20 ml.) to remove urea and electrolytes, and the adsorbed aromatic compounds were eluted from it with aq. 50% (v/v) acetone (3 × 40 ml.). The eluate was concentrated to 1 ml. at 35° *in vacuo*. A portion of this (0·2 ml., containing about 0·3 μc) was then chromatographed on Whatman no. 1 paper, first in solvent *A* and then at right-angles in solvent *B*. A further portion of the radioactive urine (0·05 ml., 0·015 μc) was applied directly to Whatman no. 1 paper and chromatographed as before. Normal rat urine was similarly treated with charcoal and the concentrated eluate from it was chromatographed as above. The chromatograms and radioautographs of the urine from rats dosed with indole revealed the presence of several metabolites, but most of the radioactivity was accounted for by six metabolites, namely indoxyl sulphate, indoxyl glucuronide, *N*-formylanthranilic acid, 5-hydroxyoxindole sulphate, 5-hydroxyoxindole glucuronide and an unidentified metabolite (see Table 1 for R_F values).

Indoxyl sulphate. This was identified by its R_F values, its strong blue fluorescence in u.v. light and by colour reactions. With Ehrlich's reagent followed by aq. saturated sodium acetate it gave a red colour, with a strong orange fluorescence in u.v. light (the fluorindal reaction), which is indicative of indoxyl derivatives. With acidified potassium persulphate it gave a blue colour due to the formation of indigotin, and with 0·1% (w/v) isatin in N-HCl followed by heating at 110° for 2 min. it gave the magenta colour of indirubin. These reactions were similar to those given by synthetic potassium indoxyl sulphate, and a mixture of the radioactive metabolite and the unlabelled synthetic indoxyl sulphate could not be separated by paper chromatography in the six solvents *A*–*F*.

Indoxyl glucuronide. This was identified by its R_F values and colour reactions (Jepson, 1960). It gave the same

colour reactions as indoxyl sulphate with acidified potassium persulphate and isatin, a positive fluorindal reaction, and a positive naphtharesorcinol reaction for glucuronic acid. Since all indoxyl derivatives give a similar blue colour (due to indigotin) on treatment with acids alone, the effect of β-glucuronidase on the metabolite was studied. The urine (12ml.) of a rat given indole was applied as a strip on four 50cm.-long sheets of Whatman 3MM paper and chromatographed in solvent *A*. The indoxyl glucuronide area from each sheet was eluted with water and purified by two-dimensional chromatography on Whatman 3MM paper in solvents *A* and *B*. The purified material was eluted with water (25ml.) and the eluate concentrated *in vacuo* to 3ml. A portion (1ml.) of the eluate was incubated with 5ml. of 5mM-acetate buffer, pH3·95, and 240 Fishman units (Talalay, Fishman & Huggins, 1946) of β-glucuronidase (0·8mg.) (Koch–Light Laboratories Ltd.) at 37° for 4hr. Paper chromatography of the material incubated with β-glucuronidase and of an ether extract of the incubated material showed that only indigotin and indirubin were present, indicating that the metabolite was a glucuronide of a precursor of these substances, most probably indoxyl glucuronide. Chromatography of the metabolite similarly incubated at pH3·95 without β-glucuronidase showed only the presence of the original conjugate.

Anthranilic acid. This was identified by its R_F values, colour reactions and its strong blue fluorescence in u.v. light. Since the 2-^{14}C atom of the radioactive indole would be eliminated in forming anthranilic acid, this metabolite was not radioactive and therefore was not detectable in radioautographs. Although this compound was also present in the chromatograms of normal rat urine, the chromatograms of the urine of rats dosed with indole showed a larger spot. The urine obtained from the rats given a total of 0·3g. of indole was acidified to pH2 and continuously extracted with ether for 5hr. The extract was concentrated to 5ml. and then purified by two-dimensional chromatography on Whatman 3MM paper in solvents *A* and *B*. The purified material was eluted with water and 2mg. of authentic anthranilic acid was added to the eluate. Chromatography of this material in six different solvents *A–F* always produced only a single spot with the R_F values of anthranilic acid.

N-Formylanthranilic acid. This was readily detected by its blue fluorescence in u.v. light and was identified by its R_F values and colour reactions. The latter are similar to those given by anthranilic acid but develop more slowly. Since the 2-^{14}C atom of the indole nucleus is still present in *N*-formylanthranilic acid, it was readily detected on radioautographs. It was present together with anthranilic acid in the ethereal extract of urine from rats given indole and was separated and purified by the chromatographic procedure used for the purification of anthranilic acid. However, repeated chromatography caused slight hydrolysis and *N*-formylanthranilic acid and anthranilic acid were always present together in chromatograms.

The *N*-formylanthranilic acid was eluted from the chromatogram with ethanol. The eluate was evaporated to dryness *in vacuo* at 37° and the residue was refluxed for 15min. in 0·1N-HCl. Paper chromatography of an ethereal extract of the hydrolysed material in solvents *A* and *B* revealed only a single spot, which from its R_F values and colour reactions was identical with anthranilic acid.

5-Hydroxyoxindole sulphate. The radioactive spot on two-dimensional paper chromatograms, with R_F values 0·35 and 0·21 in solvents *A* and *B* respectively, appeared as a dark spot when examined in u.v. light. With Ehrlich's reagent it gave an orange colour after 48hr., which suggested that the pyrrole ring of the indole molecule had been modified. The fluorindal reaction was negative and no blue colour was obtained when the compound was treated with 0·1% (w/v) isatin in N-HCl and heated at 110° for 2min. This indicated that the compound was not an indoxyl derivative. With Gibbs's reagent the compound gave a red colour, which suggested that it was not a 4- or 7-hydroxyindole derivative since these would probably give a blue colour with this reagent. No colour was obtained on diazotization and coupling with *N*-(1-naphthyl)ethylenediamine dihydrochloride, showing the absence of free aromatic amino groups. When the paper was treated with Cl_2 and then sprayed with a solution of starch and KI (Rydon & Smith, 1952) a blue colour was obtained, suggesting the presence of an amide group, but after treatment with Altman's reagent [4% (w/v) *p*-dimethylaminobenzaldehyde in acetic anhydride] and heating at 140° for 5min. no orange colour was obtained, showing that the amide group was not present in a glycine conjugate. No blue colour was obtained with indoxyl sulphuric acid, and 2,4-dinitrophenylhydrazine did not give an orange colour, showing that the compound is not a simple derivative of isatin. Treatment with rhodizonic acid–acid $BaCl_2$ (Parke, 1960) showed that the compound was a sulphate ester.

The urine obtained from ten rats, each dosed orally with indole (30mg.), was evaporated *in vacuo* at 37°. The resultant gum was extracted with three 100ml. portions of methanol–light petroleum (b.p. 40–60°) (3:1, v/v) and the extract evaporated to dryness *in vacuo* at 37°. The residue was dissolved in water (5ml.) and the solution chromatographed on Whatman 3MM paper in solvent *B*. The material of R_F 0·21 was eluted with water and purified by further chromatography first in solvent *B* and then in solvent *A*. The purified material in water (5ml.) was acidified to pH2 with 2N-HCl and refluxed under N_2 for 1hr. The hydrolysed material was continuously extracted with ether and the extract dried (anhydrous Na_2SO_4). Chromatography of the extract on Whatman no. 1 paper in solvents *A* and *B* revealed a single new spot, which was found to be chromatographically identical with a product obtained by the oxidation of oxindole with the ascorbic acid–iron hydroxylating system of Udenfriend, Clark, Axelrod & Brodie (1954). Chromatography on paper in solvents *A–F* and colour reactions with Ehrlich's and Gibbs's reagents and alkaline diazotized sulphanilic acid did not distinguish this compound from 5- or 6-hydroxyoxindole. A separation of 5- and 6-hydroxyoxindole, however, could be effected by thin-layer chromatography on alumina in solvent *G* and the hydrolysis product was finally identified as 5-hydroxyoxindole, the original metabolite being 5-hydroxyoxindole sulphate (see Table 2).

5-Hydroxyoxindole glucuronide. The radioactive spot with R_F values 0·07 and 0·20 in solvents *A* and *B* appeared as a dark spot in u.v. light. The colour reactions were identical with those given by 5-hydroxyoxindole sulphate except that the rhodizonic acid–acid $BaCl_2$ test for ethereal sulphate was negative. The naphtharesorcinol test for glucuronic acid was negative at this stage.

The rat urine extract that had been used for the chromatographic separation of 5-hydroxyoxindole sulphate was also

used for that of 5-hydroxyoxindole glucuronide. The band of R_F 0·0–0·15 of the paper chromatogram developed in solvent *A* was cut out and eluted with water (5ml.). The eluate was applied to a column (20cm.×0·6cm.) of the anion-exchange resin De-Acidite G (X2–3; 100–200mesh) prepared in the formate form. The column was washed with water (50ml.) and the metabolite was eluted with water saturated with SO_2 (50ml.), followed by water until the eluate was nearly neutral (100ml.). The eluate was concentrated *in vacuo* to 5ml., and was shown to give a strongly positive naphtharesorcinol test for glucuronic acid and a single spot of R_F values 0·07 and 0·20 when chromatographed on Whatman no. 1 paper in solvents *A* and *B* respectively.

The eluate was refluxed with conc. HCl (0·2vol.) for 1hr. under N_2. A sample of the hydrolysed solution was continuously extracted with ether for 4hr. and the extract dried over anhydrous Na_2SO_4. Chromatography of the ethereal extract on thin-layer plates of alumina in solvent G (Table 2) showed one spot, which was identical with 5-hydroxyoxindole. The remainder of the hydrolysed solution was absorbed on a column (7cm.×1cm.) of activated charcoal (Ultrasorb S.C. 120/240; British CarboNorit Ltd.) (10g.). The column was washed with water until neutral, and then eluted with 30ml. of aq. 5% (v/v) ethanol to remove sugar derivatives. The eluate, after concentration *in vacuo* at 37° to 2ml., was chromatographed on Whatman no. 1 paper in three different solvent systems, namely propan-2-ol–water (4:1, v/v), propan-2-ol–butan-1-ol–water (7:1:2, by vol.) and butan-1-ol–acetic acid–water (12:3:5, by vol.). The three chromatograms were developed by spraying with the naphtharesorcinol reagent and then heated at 100° for 10min. Two spots corresponding in R_F values to D-glucuronic acid and D-glucuronolactone were detected on all three chromatographs and it was therefore concluded that the original metabolite was the glucuronide of 5-hydroxyoxindole.

Oxindole and isatin. Although these two compounds are possible metabolites of indole, they were not detected on paper chromatograms or radioautographs of the urine of rats dosed with [2-^{14}C]indole. Since 5-hydroxyoxindole is a metabolite of indole, then its precursor might be oxindole, for the feeding of oxindole to rats results in the excretion of 5-hydroxyoxindole and its conjugates (see Table 3). Both oxindole and isatin are readily converted into coloured bis-indoles. Isatin is readily reduced to the yellow isatan, and can condense with oxindole to give isatan or the red isoindigotin. Oxindole or isatin can condense with indoxyl to give indirubin. When oxindole (0·1mg./ml.) is added to normal rat urine it can be partly recovered by $CHCl_3$ extraction and subsequent thin-layer chromatography on alumina with solvent *G*. Isatin (0·1mg./ml.), however, was largely recovered as a yellow pigment, probably isatan. When added to the fresh urine of rats dosed with indole, both could be detected in thin-layer chromatograms of $CHCl_3$ extracts of the urine, but attempts at purification by elution and further chromatography resulted in their conversion into pigments including isatan and indirubin.

The urine (pH8·0–8·2) of six rats, which had collectively received orally 90mg. of indole in arachis oil, was adjusted to pH3 with 0·1N-HCl as soon as it was passed during 24hr. Each sample was immediately extracted twice with $CHCl_3$ (0·5vol.), and the combined extracts (30ml.) were dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The concentrate was chromatographed as a band on a thin-layer plate of alumina in $CHCl_3$ and run with reference spots (20μg.) of isatin and oxindole. Many coloured bands were formed, the most prominent of which were the following (R_F values given in parentheses): deep blue (origin); buff (0·1–0·12); blue (0·12); violet (0·14); yellow, several bands (0·14–0·35); violet (0·35–0·65); violet–grey (0·65–0·80); blue (0·80–0·95); buff (0·95). The areas (R_F 0·1–0·22 and 0·4–0·6) containing isatin and oxindole respectively were cut out and eluted with $CHCl_3$ (2×2ml.). After centrifuging, the extract was concentrated and chromatographed on thin-layer alumina in solvent *G*. The material from the first area gave bands of R_F 0·21 (isatin), 0·38 (isatan) and 0·40 (indirubin). Further chromatography of this isatin band after elution resulted in the complete loss of the isatin, and only isatan and indirubin was found. Similar treatment of the second area above yielded several coloured bands on rechromatographing, including indirubin, but there was one colourless spot (R_F 0·52, oxindole), which gave a red colour with Gibbs's reagent and strongly absorbed u.v. light. Elution of this spot and further chromatography resulted in its disappearance and the simultaneous appearance of coloured bands of bis-indoles.

These experiments show that oxindole and isatin are detectable as metabolites of indole in fresh urine. It is probable that these compounds are reasonably stable in urine, but as soon as the urine is acidified indoxyl is released from its conjugates and the formation of bis-indoles can occur. Oxindole and isatin also disappear when chromatographed in small quantities. These points had to be carefully considered when isotope dilutions were carried out for oxindole and isatin.

Isotope dilution procedures

Total indoxyl. Carrier potassium indoxyl sulphate (0·5g.) was added to urine (6ml.) or bile (2ml.) or faecal homogenate (30ml.). Isatin (0·35g.) and 5N-H_2SO_4 (40ml.) were then added and the mixture was heated under reflux for 4·5hr. After cooling the indirubin formed was filtered off, washed with water, with 2N-NaOH and again with water, and finally recrystallized from aqueous acetone to constant specific activity. The indirubin was then converted into its oxime (Lucius & Brüning, 1914–1916), which was recrystallized from aqueous acetone to constant specific activity (m.p. 246°, decomp.).

Indoxyl sulphate. A solution of potassium indoxyl sulphate (0·5g. in 20ml. of water) was added to urine (6ml.) or bile (2ml.) or faecal homogenate (30ml.). The mixture was then poured into a solution of 5-aminoacridine hydrochloride (0·5g. in 20ml. of hot water). After cooling, the 5-aminoacridine salt of indoxyl sulphate was filtered off, washed with cold water, dried and recrystallized from hot water to constant specific activity (m.p. 215°, decomp.).

N-Formylanthranilic acid. *N*-Formylanthranilic acid (0·5g.) dissolved in saturated $NaHCO_3$ (25ml.) was added to urine (6ml.) and reprecipitated by acidifying with 2N-HCl. It was filtered off, washed with water and then hydrolysed by heating in a micro-distillation apparatus with H_3PO_4 (sp.gr. 1·75) (1ml.) and water (1ml.). The radioactive formic acid liberated was then distilled into a receiver cooled to −30°, and neutralized with 10N-NaOH and finally adjusted to pH6·4 with 0·01N-HCl. The solution was then refluxed for 0·5hr. with 0·8g. of 4-bromophenacyl bromide and 10ml. of ethanol. After cooling, the crystalline

4-bromophenacyl formate was filtered, dried and recrystallized from light petroleum (b.p. 60–80°) to constant specific activity (m.p. and mixed m.p. 95°).

Total 5-hydroxyoxindole. The carrier (0·3g.) was added to urine (6ml.) or bile (2ml.) and the mixture refluxed with 12ml. of 3N-HCl for 1·5hr. under N_2. The solution was cooled and continuously extracted with ether for 48hr. The extract was dried over anhydrous Na_2SO_4, evaporated to dryness *in vacuo* and then recrystallized from water (charcoal) to constant activity (m.p. 269°, decomp.). This material was further purified by chromatography on thin-layer plates of alumina in solvent *G*. The 5-hydroxyoxindole found at R_F 0·6 was cut out, eluted with methanol and the eluate evaporated to dryness *in vacuo*. The 5-hydroxyoxindole purified in this way contained no 6-hydroxyoxindole and had the same specific activity as the material crystallized from water.

Total 6-hydroxyoxindole. 6-Hydroxyoxindole (0·3g.) was added to urine or bile, and the procedure described for 5-hydroxyoxindole was followed. The recrystallized 6-hydroxyoxindole (m.p. 265°, decomp.) contained considerable radioactivity, which was found to be due to the presence of traces of the 5-isomer. Chromatography of the recrystallized material on thin-layer plates of alumina in solvent *G* separated these isomers. The 6-hydroxyoxindole was eluted with methanol and the specific activity of material purified in this way was negligible.

Indole. Carrier indole (0·5g.) was added to urine (6ml.) or homogenized faeces (60ml., equivalent to 30g. of faeces) and refluxed with 200ml. of water for 0·5hr. The mixture was then steam-distilled and the distillate (500ml.) extracted with ether (3×50ml.). The combined extracts were dried (anhydrous Na_2SO_4) and evaporated to dryness *in vacuo* at room temperature. The residue was dissolved in CCl_4 (1–2ml.) and the solution chromatographed on a column (20cm.×1cm.) of alumina (100g.; Spence grade H) previously deactivated by shaking for 24hr. with 10% (v/v) acetic acid. The material was eluted with CCl_4, and the first 500ml. of eluate, which contained most of the indole, was evaporated to dryness at room temperature *in vacuo*. The residue dissolved in 5ml. of ethanol was then treated with a hot solution of picric acid (1g.) in ethanol (20ml.). On cooling, the crystalline indole picrate was filtered off and recrystallized from ethanol to constant specific activity (m.p. 184°).

Oxindole. Oxindole (0·5g.) was added to a mixture of the urine (2ml.) and 2N-HCl (25ml.) and the whole refluxed under N_2 for 0·5hr. The solution was cooled and continuously extracted with ether for 4hr. After drying over anhydrous Na_2SO_4, the extract was evaporated to dryness. The residue in benzene (2ml.) was chromatographed on a column (20cm.×1cm.) of alumina (20g.) partially deactivated with 10% acetic acid. The column was eluted with benzene, and the first 150ml. of eluate, which contained most of the oxindole, was evaporated to dryness *in vacuo*. The residue of oxindole, m.p. 128°, was repeatedly recrystallized to constant specific activity from water (charcoal).

Isatin. Isatin (0·3g. in 100ml. of water) was added to urine (2ml.) and the solution left for 5min. to equilibrate. The solution was extracted with $CHCl_3$ (3×50ml.) and the combined extracts were dried (anhydrous Na_2SO_4) and evaporated to dryness *in vacuo*. The residue was chromatographed on thin-layer chromatoplates of alumina in $CHCl_3$, and the band of isatin (R_F 0·0–0·25) was removed and eluted with methanol. The eluate was evaporated to dryness *in vacuo* and the residue of isatin recrystallized from water and then benzene–methanol (4:1, v/v) to constant specific activity, m.p. 211°. The purified isatin (0·15g. in 50ml. of hot water) was heated on a water bath for 20min. with phenylhydrazine hydrochloride (0·16g.) and sodium acetate (0·1g.). The mixture was cooled, and the product of isatin-3-phenylhydrazone filtered off, washed with water and recrystallized from methanol to constant specific activity (m.p. 212°). The isatin-3-phenylhydrazone was further purified by chromatography on thin-layer chromatoplates of alumina first in $CHCl_3$ (R_F 0·92) and then at right-angles in solvent *G* (R_F 0·85). The purified material was eluted with methanol and determination of its specific activity showed that this was unchanged.

Metabolism of indole and oxindole in vitro

Aerobic oxidation of indole. The microsome plus supernatant fraction (10000*g*) of liver homogenates of female albino rats containing the equivalent of 0·25g. of liver/ml. was prepared (see Creaven, Parke & Williams, 1965). Indole (112μmoles or 13·1mg. plus 250mg. of Tween 80 to solubilize it) with 28ml. of the 10000*g* supernatant, 14ml. of 0·1M-phosphate buffer, pH7·4, glucose 6-phosphate (168μmoles), nicotinamide (280μmoles) and NADPH (7μmoles, as the tetrasodium salt), in a total volume of 56ml. of 1·15% (w/v) KCl, was incubated in fourteen 100ml. beakers in air at 37° for 1hr. in a Mickle shaking incubator. The incubation mixtures were combined, 1ml. of 10N-HCl was added and the mixture was extracted with ether (2×100ml.). The extract was dried over anhydrous Na_2SO_4, concentrated *in vacuo* at room temperature to 2ml. and then chromatographed on paper in solvents *A* and *B*.

Seven spots were detected on the chromatograms, and by comparison with the R_F values and colour reactions of authentic materials they were identified as indole, indigotin, indirubin, oxindole, *N*-formylanthranilic acid and anthranilic acid. The seventh spot had R_F values 0·71 and 0·73 in solvents *A* and *B* respectively and gave a yellow colour with Ehrlich's reagent and purple fluorescence in u.v. light, but it was not identified.

The ethereal extract of a similar incubation of indole with rat-liver 10000*g* supernatant preparation was dried over anhydrous Na_2SO_4 and evaporated to dryness *in vacuo*. The residue was dissolved in 2ml. of benzene and chromatographed on a column (20cm.×0·6cm.) of alumina (Spence grade H, deactivated with 10% acetic acid). The column was developed with benzene (30ml.), to elute the unchanged indole, indigotin and indirubin, and then with benzene–ether (10:1, v/v) (30ml.) to elute oxindole, which was detected by the red colour it gave with Gibbs's reagent. The solution of oxindole was evaporated to dryness *in vacuo*, and the residue, dissolved in butan-1-ol (3ml.), was heated in a water bath for 5hr. with 1·0ml. of a solution of *p*-dimethylaminocinnamaldehyde in butan-1-ol (250μg./ml.) and 1 drop of pyridine. The solution was then diluted to 25ml. with butan-1-ol and the u.v.-absorption spectrum of the solution determined. The absorption spectrum ($\lambda_{max.}$ 460) of the condensation product of *p*-dimethylaminocinnamaldehyde with the oxindole formed enzymically from indole was identical with that prepared similarly from authentic oxindole ($\lambda_{max.}$ 460mμ).

Anaerobic oxidation of indole. The microsomal oxidation of indole was also studied anaerobically. A 10000*g* supernatant preparation (28ml.) of rat-liver homogenate with phosphate buffer, pH 7·4, glucose 6-phosphate, nicotinamide and NADPH, in the same quantities as before, was distributed equally in 12 Thunberg tubes. The tubes were evacuated and then flushed with N_2 three times before finally filling with N_2 and introducing the substrate, indole (13·1mg. plus 250mg. of Tween 80). The tubes were then incubated at 37° for 1hr. in a Mickle shaking incubator. The incubation mixtures were combined, 1ml. of 10N-HCl was added and the solution was extracted with ether (2×100ml.). The extract (dried over anhydrous Na_2SO_4) was concentrated *in vacuo* at room temperature to 2ml. and the residue chromatographed on paper in solvents *A* and *B*.

Two spots were detected on the chromatograms and, by comparison with the R_F values and colour reactions of authentic compounds, these were shown to be indole and oxindole. Indigotin, indirubin, *N*-formylanthranilic acid and anthranilic acid were not detected.

Oxindole. Oxindole (112μmoles) was substituted for indole plus Tween 80 in the above incubation mixture and the whole incubated at 37° for 1hr. in air. Extracts were prepared as above and chromatographed on paper in solvents *A*, *B* and *C*. Four spots were detected, which were identified as oxindole, 5-hydroxyoxindole, anthranilic acid and *o*-aminophenylacetic acid. The identity of 5-hydroxyoxindole was confirmed by chromatography of the ethereal extract on thin-layer plates of alumina in solvent *G*. 6-Hydroxyoxindole was not detected.

Metabolism of indole metabolites

The metabolism in the rat of some of the possible metabolites of indole, namely indoxyl, oxindole, *N*-formylanthranilic acid, anthranilic acid, 5- and 6-hydroxyindole and 5-, 6- and 7-hydroxyoxindole, was studied qualitatively. These compounds were administered orally and samples of the 24hr. urines were chromatographed on paper in solvents *A* and *B* as described above. The excretory products in urine were detected and identified by R_F values and colour reactions (see Table 3).

Table 3. *Fate of some possible metabolites of indole in the rat*

The compounds were given orally, and the 24hr. urine was examined chromatographically on Whatman no. 1 paper. The metabolites were identified by R_F values and colour reactions (see the text). Abundance: +++, major metabolite; ++, smaller amounts; +, minor metabolite.

		Metabolites detected			
		R_F in			
Compound	Dose (mg./rat)	Solvent *A*	then solvent *B*	Identity	Abundance
Indoxyl acetate	30	0·54	0·40	Indoxyl sulphate	+++
or		0·19	0·44	Indoxyl glucuronide	+++
potassium indoxylsulphate	100	0·43	0·90	Anthranilic acid	+
		0·52	0·90	*N*-Formylanthranilic acid	+
Oxindole	30	0·35	0·21	5-Hydroxyoxindole sulphate	+++
		0·09	0·20	5-Hydroxyoxindole glucuronide	+
		0·58	0·65	5-Hydroxyoxindole	Trace
5-Hydroxyindole	20	0·49	0·40	5-Hydroxyindole sulphate	+++
		0·15	0·31	5-Hydroxyindole glucuronide	+++
		0·85	0·85	5-Hydroxyindole	+
6-Hydroxyindole	20	0·50	0·40	6-Hydroxyindole sulphate	+++
		0·23	0·34	6-Hydroxyindole glucuronide	+++
5-Hydroxyoxindole	30	0·35	0·21	5-Hydroxyoxindole sulphate	+++
		0·09	0·20	5-Hydroxyoxindole glucuronide	+
		0·58	0·65	5-Hydroxyoxindole	Trace
6-Hydroxyoxindole	30	0·36	0·21	6-Hydroxyoxindole sulphate	+++
		0·08	0·21	6-Hydroxyoxindole glucuronide	+
		0·57	0·63	6-Hydroxyoxindole	Trace
7-Hydroxyoxindole	30	0·62	0·36	7-Hydroxyoxindole sulphate	+++
		0·15	0·30	7-Hydroxyoxindole glucuronide	+
N-Formylanthranilic acid	100	0·28	0·38	Anthranilyl glucuronide	+++
		0·38	0·74	Anthranilyl glycine	++
		0·43	0·91	Anthranilic acid	++
		0·52	0·91	*N*-Formylanthranilic acid	++
Anthranilic acid	100	0·28	0·38	Anthranilyl glucuronide	+++
		0·38	0·74	Anthranilyl glycine	++
		0·43	0·91	Anthranilic acid	++

Indoxyl was administered either as indoxyl acetate (30mg.) or potassium indoxyl sulphate (100mg.). The output of indoxyl glucuronide after feeding with these compounds appeared to be greater than after feeding with indole. Small amounts of anthranilic acid and *N*-formylanthranilic acid were detected but 5-hydroxyoxindole and its conjugates were not found.

Oxindole (30mg.) gave rise to the excretion of 5-hydroxyoxindole and its conjugates, principally the sulphate. Oxindole, 6-hydroxyoxindole, *N*-formylanthranilic acid, anthranilic acid and their conjugates were not detected.

Anthranilic acid (100mg.) was excreted principally as such and as two compounds, which were identified as anthranilyl glucuronide and anthranilyl glycine, the known metabolites of anthranilic acid (Charconnet-Harding, Dalgliesh & Neuberger, 1953). The spots on the chromatogram corresponding to these compounds gave a violet colour when diazotized and coupled with *N*-(1-naphthyl)-ethylenediamine (1% in water), thus indicating a free aromatic amino group in each. A spot with R_F values 0·28 and 0·38 in solvents *A* and *B* respectively gave a positive naphtharesorcinol test for glucuronic acid, whereas a spot with R_F values 0·38 and 0·74 gave the characteristic orange colour of a glycine conjugate after spraying with a solution of *p*-dimethylaminobenzaldehyde (4% in acetic anhydride) and heating at 140° for 3min. Free glucuronic acid was also detected on the chromatograms; this could arise from anthranilyl glucuronide or possibly from an *N*-glucuronide of anthranilic acid.

N-Formylanthranilic acid (100mg.) administered orally or intraperitoneally gave similar results. Some unchanged *N*-formylanthranilic acid was excreted but the major metabolites were anthranilic acid and its glycine and glucuronide conjugates.

5- and 6-Hydroxyindole (20mg.) each gave rise to the excretion of their respective ester sulphates and glucuronides as the major metabolites. Free 5-hydroxyindole was also excreted in small amounts but free 6-hydroxyindole was not detected in the urine. 5- and 6-Hydroxyoxindole and their conjugates were not found.

5- and 6-Hydroxyoxindole (30mg.) each gave three spots on chromatograms of the urine excreted. These were identified as the corresponding ethereal sulphates, which were the major metabolites, the glucuronides and small amounts of the free hydroxyoxindoles. No spots corresponding to *N*-formylanthranilic acid or anthranilic acid were detected.

RESULTS AND DISCUSSION

Qualitative chromatographic studies of the urine of rats dosed with [2-^{14}C]indole show that the major metabolites of indole are 3-hydroxyindole sulphate and glucuronide, and that the minor metabolites are 5-hydroxyoxindole sulphate and glucuronide, *N*-formylanthranilic acid and anthranilic acid. Isotope dilution experiments showed that oxindole and isatin were also minor metabolites but these two compounds were only present, as shown by thin-layer adsorption chromatography, in the fresh urine, for they were rapidly converted into coloured bis-indole compounds in the presence of conjugates of indoxyl. The anthranilic acid found in the urine may have been formed by spontaneous decomposition of *N*-formylanthranilic acid, since the oral administration of anthranilic acid results largely in its excretion as the glucuronic acid and glycine conjugates.

When [2-^{14}C]indole (70–80mg./kg. body wt.) is administered orally to rats, the radioactivity is fairly rapidly excreted in the urine, faeces and expired air. The major route of excretion is the urine, for 75% of the dose is excreted by this route in 24hr. and nearly 80% in 2 days. About 10% is eliminated in the faeces and 2% in the expired air as $^{14}CO_2$. Relatively small amounts of radioactivity (0·4% of dose) were found to remain in the tissues 2 days after dosing (Table 4). Of the radioactivity in the urine, an average of 72% of the dose is accounted for as indoxyl sulphate (50% of the dose), indoxyl glucuronide (11%), 5-hydroxyoxindole conjugates (3·1%), isatin (5·8%), oxindole (1·4%) and *N*-formylanthranilic acid (0·5%) (Table 5). The nature of the faecal radioactivity

Table 4. *Distribution of* ^{14}C *in the excreta and tissues of rats receiving* [2-^{14}C]*indole orally*

—, Not determined.

	Percentage of dose in 2 days							
Rat no.	1	2	3	4	5	6	7	Average for rats 4, 6 and 7
Weight (g.)	230	170	180	200	200	220	240	
Dose of indole (mg./kg.) ...	76	70	80	80	74	70	64	
Dose of ^{14}C (μc)	0·5	0·34	0·5	9·0	8·2	8·8	8·8	
Urine	65·6	86·0	58·0	92·3	90·3	72·1	77·9	80·6
Faeces	12·4	11·7	9·1	7·2	3·7	15·4	10·7	11·1
Expired air	—	—	—	3·1	—	1·9	2·1	2·4
Tissues	—	—	—	0·0	0·4	0·4	0·2	0·2
Total accounted for	78·0	97·7	67·1	102·6	94·4	89·8	90·0	94·3

Table 5. *Quantitative aspects of the metabolism of* [2-^{14}C]*indole in rats*

Details of the methods used are given in the text. The dose of indole and of ^{14}C are given in Table 4, and the values quoted are for excreta collected for 2 days after dosing with indole.

	Percentage of dose of ^{14}C		
Rat no.....5	6	7
Expired air			
CO_2	—	1·9	2·1
Urine			
Indole	0·0	0·06	0·07
Total indoxyl	67·6*	60·8	59·9
Indoxyl sulphate	49·8	53·7	45·2
Indoxyl glucuronide	17·8*†	7·1†	14·7†
Total 5-hydroxyoxindole	—	3·0	3·1
Total 6-hydroxyoxindole	—	0·0‡	0·0‡
Oxindole	—	1·9	0·9
Isatin	—	5·5	6·1
N-Formylanthranilic acid	0·63	0·44	0·53
Total of above metabolites in urine	68	72	71
Total ^{14}C in urine	90	72	78
Faeces			
Indole	0·09	0·13	0·19
Total indoxyl	—	0·68	0·59
Indoxyl sulphate	—	0·43	0·37
Total of above metabolites in faeces	0·09	0·81	0·78
Total ^{14}C in faeces	3·7	15·4	10·7

* Includes isatin.
† Difference between total indoxyl and indoxyl sulphate.
‡ Limit of detection is 0·3% of dose.

Table 6. *Excretion of* ^{14}C *in the bile of rats receiving* [2-^{14}C]*indole*

Rats were cannulated as described in the text and given [2-^{14}C]indole orally. Bile was collected for 2 days.
—, Not determined.

	Percentage of dose of ^{14}C in the 2-day bile			
Rat no.	8	9	10	11
Weight (g.)	200	200	200	210
Dose of indole (mg./kg.)	21	21	63	49
Dose of ^{14}C (μc)... ...	2·3	2·3	7·3	5·9
Total indoxyl	—	—	0·85	0·78
Indoxyl sulphate	—	—	0·84	0·75
5-Hydroxyoxindole	—	—	0·52	0·60
Total of above	—	—	1·37	1·38
Total ^{14}C in bile	5·4	7·1	4·65	5·2 (average 5·6)

(10% of dose) has not been elucidated, but indole (0·2% of dose) and indoxyl sulphate (0·4% of dose) are only minor components of it (Table 5). Radioactivity is also excreted in the expired air as $^{14}CO_2$ (2% of the dose), so that at least an equivalent amount of indole must have been metabolized into non-radioactive metabolites by scission of the pyrrole ring and loss of the 2-^{14}C atom. Neither indole nor any other volatile metabolite was detected in the expired air. When [^{14}C]indole is given orally to biliary-cannulated rats an average of 5% of the administered radioactivity is eliminated in the bile in 48 hr., of which 0·8% of the dose is indoxyl sulphate and 0·6% is conjugated 5-hydroxyoxindole (Table 6).

To elucidate the routes of metabolism of indole in the rat, studies were made of the metabolism of indole and its possible metabolites *in vivo* and *in vitro*. When indoxyl is given orally, it gives rise to the excretion of *N*-formylanthranilic acid and

anthranilic acid, whereas oxindole gives rise to 5-hydroxyoxindole and its conjugates. When 5- and 6-hydroxyindole are administered to rats they are excreted in the urine as the sulphate and glucuronic acid conjugates, and oxidation of the pyrrole ring to give hydroxyoxindoles does not occur. When indole is incubated aerobically with rat-liver microsomes plus supernatant, there are formed indigotin, indirubin, oxindole, *N*-formylanthranilic acid and anthranilic acid, whereas under anaerobic conditions only oxindole is formed. The occurrence of indigotin and indirubin in the incubation mixtures suggests that indoxyl and possibly isatin are formed from indole under aerobic conditions. When oxindole is incubated aerobically with similar rat-liver preparations, 5-hydroxyoxindole, anthranilic acid and *o*-aminophenylacetic acid, but not *N*-formylanthranilic acid, are formed. It thus appears that initially indole undergoes two separate hydroxylations, one in the 3-position to give indoxyl and one in the 2-position to give oxindole. The oxidation in the 2-position can proceed under aerobic or anaerobic conditions and possibly the oxygen introduced is derived from water. The oxidation in the 3-position, however, occurs only under aerobic conditions, and presumably, like other microsomal hydroxylations of foreign compounds, it requires molecular oxygen and NADPH.

The initial oxidation of indole occurs mainly in the 3-position, the position of highest electron density, and to a smaller extent in the 2-position, the position of second highest electron density. The indoxyl formed is now mainly conjugated with sulphate and glucuronic acid and excreted. However, some indoxyl is further oxidized to formylanthranilic acid, possibly via 2,3-dihydroxyindole, dioxindole and isatin. To a small extent formylanthranilic acid probably yields anthranilic acid and formate, the latter probably being the source of the $^{14}CO_2$ in the expired air. The oxindole formed does not appear to be able to conjugate owing to lack of suitable groupings. It therefore undergoes further hydroxylation in the 5-position to 5-hydroxyoxindole. The hydroxylation of oxindole in the 5-position is to be expected since this position is *para* to the NH group. 5-Hydroxyoxindole is then largely disposed of by conjugation with sulphate and glucuronic acid. The experiments with oxindole *in vitro* suggest that it could break down to *o*-aminophenylacetic acid and possibly to anthranilic acid. Most of these suggested pathways require further detailed examination.

The metabolism of indole is reminiscent of that of quinoline, in which the major initial hydroxylations occur in the 2- and 3-positions. 2-Hydroxyquinoline or 2-quinolone is then further hydroxylated to 6-hydroxy-2-quinolone, position 6 in quinoline being the equivalent of position 5 in indole (see Smith & Williams, 1955; Sax & Lynch, 1964).

The metabolism of indole as elucidated so far is shown in Scheme 1.

This work was supported by a grant from Shell Research Ltd., Shell Centre, London.

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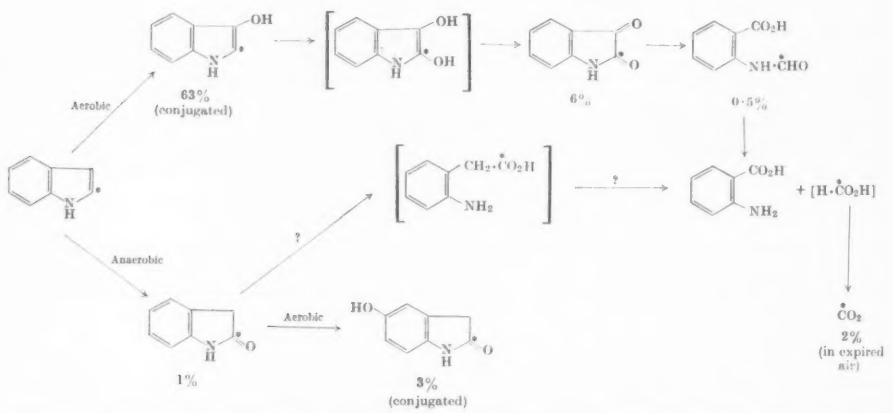

Scheme 1. Hypothetical metabolites are in brackets and the percentage of dose found in the urine (except CO_2) as a particular metabolite is given under its formula.

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The Interpretation of Non-Hyperbolic Rate Curves for Two-Substrate Enzymes

A POSSIBLE MECHANISM FOR PHOSPHOFRUCTOKINASE

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(*Received* 8 *June* 1965)

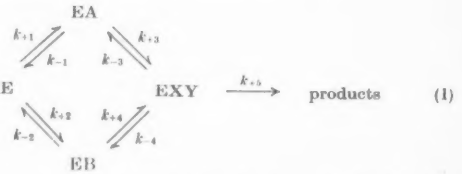
1. A theoretical appraisal of the alternative pathway mechanism for a two-substrate enzyme shows that this mechanism is capable of giving rise to apparent substrate inhibition or substrate activation (Dalziel, 1958). It has now been shown that these phenomena may occur simultaneously in the following ways. With certain relationships between the kinetic parameters and the constant concentration of one substrate, A, the plot of initial rate, v, against the concentration of the other substrate, B, may show substrate 'activation' at low concentrations of B and substrate 'inhibition' at high concentrations of B. In other circumstances the plot of v against [B], with [A] constant, may be sigmoid (substrate activation), whereas the plot of v against [A], with [B] constant, may pass through a maximum (substrate inhibition). 2. Kinetic data for phosphofructokinase are of the latter type and it is suggested that the mechanism of this enzyme may involve a kinetically preferred pathway. It is emphasized that the phenomena of substrate inhibition and activation need not necessarily involve more than one binding site for each substrate on the enzyme molecule, nor more than one monomer per molecule.

The current interest in regulatory enzymes, many of which exhibit curves of initial velocity against substrate concentration that are either sigmoid or pass through maxima, stimulated a search for models of enzyme mechanism that would explain such kinetic data.

Where sigmoid curves have been found they have often been interpreted in terms of co-operative sub-unit interactions of the type described by Adair (1925) for haemoglobin. Though this is probably correct in many cases, Atkinson & Walton (1965) have pointed out that '... sigmoid rate curves... do not necessarily require sub-unit aggregation or more than one catalytic site per molecule'. Further, Dalziel (1957) has shown that an alternative pathway mechanism for a two-substrate enzyme may in certain cases give rise to substrate activation.

Similarly, where rate curves have been found to pass through a maximum it has often been concluded that a second binding site for substrate is necessary to explain the substrate inhibition at high concentrations (e.g. Alberty, 1956). Such a conclusion is only one of several possible ones, as has been shown previously (Dalziel, 1957, 1958; Theorell & McKinley-McKee, 1961; Raval & Wolfe, 1963; Massey & Veeger, 1963).

The purpose of the present paper is to investigate the general mechanism for a two-substrate enzyme and to determine under what special conditions it may give rise to substrate activation (a sigmoid rate curve) or substrate inhibition (a rate curve with a maximum) without postulating more than one binding site for each substrate on the enzyme molecule. The general mechanism considered (see eqn. 1) is one where each substrate can combine with the free enzyme to give a binary complex, the binary complexes each then combining with the other substrate to give the same ternary complex, which breaks down to products. Alternative pathways to the ternary complex are thus available. Dalziel (1958) has pointed out that when treated by the steady-state method '... substrate "activation" and "inhibition" are inherent in this mechanism'.



(1)

$$k_{+1}\,k_{-2}\,k_{+3}\,k_{-4} = k_{-1}\,k_{+2}\,k_{-3}\,k_{+4}$$

The initial rate in the absence of products and in the presence of a constant concentration, b, of the second substrate, B, is given by:

$$v = \frac{v'}{e} = \frac{ia^2+ja}{k+la^2+ma} \qquad (2)$$

where a is the concentration of A, e is the total enzyme concentration and i, j, k, l and m are functions of b and the rate constants for the various steps shown in eqn. (1) (Ingraham & Makower, 1954; Dixon & Webb, 1964).

Differentiation of the rate eqn. (2) gives:

$$\frac{dv}{da} = \frac{a^2(im-jl)+2kia+kj}{(k+la^2+ma)^2} \qquad (3)$$

There will be maxima or minima in the plot of v against a when $dv/da = 0$, i.e. when $a = \pm\infty$ or when the numerator of eqn. (3) is zero. In the latter case the roots are given by:

$$a = -\frac{ki \pm \sqrt{k^2i^2 - kj(im-jl)}}{(im-jl)}$$

These roots will be negative and therefore of no practical interest, except when $im < jl$, when there will be one positive root and one negative root.

The second differential of the initial rate is given by:

$$\frac{1}{2}\cdot\frac{d^2v}{da^2} = -\frac{l(im-jl)a^3 - 3kila^2 - 3kjla + k(ki-mj)}{(k+la^2+ma)^3} \qquad (4)$$

This shows that points of inflexion will be present in the plot of v against a when $a = \pm\infty$ or when the numerator of eqn. (4) is zero, i.e. when:

$$a^3 + \frac{3ki}{(im-jl)}\cdot a^2 + \frac{3kj}{(im-jl)}\cdot a + \frac{k}{l}\cdot\frac{(mj-ki)}{(im-jl)} = 0 \qquad (5)$$

The roots of eqn. (5) will, once more, be negative and of no practical interest, except when either of the following conditions is met:

Condition (i): $ki > mj$ and $im > jl$

Condition (ii): $im < jl$

In the second condition, from the earlier consideration, there will also be a maximum at a positive value of a. If, at the same time, $ki < mj$ there will be a single point of inflexion at a positive value of a (ii a), whereas if $ki > mj$ there will be two positive points of inflexion (ii b). If $ki = mj$ there will be one point of inflexion at the origin and one at a positive value of a. The expected rate curves will therefore have the appearance shown in Fig. 1.

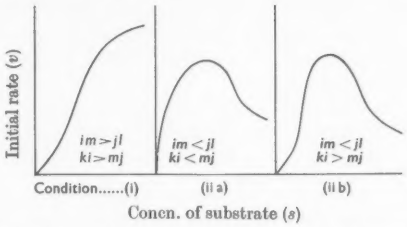


Fig. 1. Shapes of the initial rate curves for the alternative pathway mechanism (eqns. 1 and 2 in the text) with various relationships among the constants i, j, k, l and m of eqn. (2). Botts (1958) has also described the general shape of the curves that may arise from eqn. (2), in connexion with various single-substrate enzyme models.

Fig. 1 (ii b) indicates that this mechanism could give rise to a rate curve showing substrate activation at low substrate concentrations and substrate inhibition at high substrate concentrations. Each of the constants i, j, k, l and m is itself a very complex function of the rate constants and of b (see Dixon & Webb, 1964), so that conditions (i) and (ii) are extremely complex in terms of the rate constants and b. It has not been possible to reduce these conditions to a form in which obvious relationships among the rate constants can be seen. It appears that for either condition k_{+5} should be large, that part of condition (i) is $k_{+1} > k_{+4}$ and that part of condition (ii) is $k_{+2} > k_{+3}$. However, a number of hypothetical cases were inspected to see whether any plausible combinations of the rate constants would meet either of the above conditions for points of inflexion. As would be expected, no case where breakdown of EXY to products is rate-limiting (i.e. 'rapid-equilibrium' or Michaelis–Menten–Haldane kinetics) will meet either condition; nor will any steady-state situation, where both routes to EXY (see eqn. 1) are equally favoured, or where one route is obligatory.

On the other hand, the following situation meets condition (i). Let the values of the rate constants in eqn. (1) be as follows:

$$k_{+1} = k_{+3} = 10^5;\ k_{+2} = k_{+4} = 10^4;\ k_{+5} = 10^6$$
$$k_{-1} = k_{-3} = 10^{-1};\ k_{-2} = k_{-4} = 10^{-2}$$

(the rate constants and concentrations have not been given any dimensions, but any consistent set of dimensions would be acceptable). The reaction now has a kinetically preferred pathway:

$$E \xrightarrow{A} EA \xrightarrow{B} EXY \longrightarrow \text{products}$$

Table 1. *Numerical values of the quantities involved in conditions* (i) *and* (ii) (*see the text*) *for various values of* b, *in the case of the hypothetical enzyme described in the text*

b	ki	mj	im	jl
10^{-2}	$1{\cdot}00\times10^{29}$	$1{\cdot}00\times10^{28}$	$1{\cdot}00\times10^{31}$	$1{\cdot}00\times10^{30}$
10^{-3}	$1{\cdot}00\times10^{26}$	$1{\cdot}01\times10^{25}$	$1{\cdot}00\times10^{29}$	$1{\cdot}01\times10^{28}$
10^{-4}	$1{\cdot}02\times10^{23}$	$1{\cdot}13\times10^{22}$	$1{\cdot}02\times10^{27}$	$1{\cdot}11\times10^{26}$
10^{-5}	$1{\cdot}21\times10^{20}$	$2{\cdot}52\times10^{19}$	$1{\cdot}20\times10^{25}$	$2{\cdot}10\times10^{24}$
10^{-6}	$4{\cdot}00\times10^{17}$	$3{\cdot}60\times10^{17}$	$3{\cdot}00\times10^{23}$	$1{\cdot}20\times10^{23}$
10^{-7}	$1{\cdot}21\times10^{16}$	$2{\cdot}33\times10^{16}$	$2{\cdot}10\times10^{22}$	$1{\cdot}11\times10^{22}$

but the other pathway is not forbidden:

$$\mathrm{E} \xrightarrow{\mathrm{B}} \mathrm{EB} \xrightarrow{\mathrm{A}} \mathrm{EXY} \longrightarrow \text{products}$$

The dissociation constants K_1, K_2, K_3 and K_4 for the scheme (see eqn. 1) are all equal to 10^{-6}, so that there is no thermodynamic preference for any one step.

If b is set at a constant value, the values of i, j, k, l and m may be calculated from the equations set out by Dixon & Webb (1964). These values lead to the relationships shown in Table 1, and these satisfy condition (i) in all cases, except when $b=10^{-7}$. This demonstrates that for values of $b \geqslant 10^{-6}$ there will be a point of inflexion in the graph of v against a, which is shown for $b=10^{-3}$ in Fig. 2(*a*). The points have been calculated from eqn. (2), by substituting the calculated values of i, j, k, l and m. There is a point of inflexion at $a=4{\cdot}6\times10^{-4}$.

If the points shown in Fig. 2(*a*) had been obtained experimentally and had been assumed to follow the equation:

$$v = V_{\mathrm{max.}} \cdot \frac{a^n}{k+a^n}$$

the maximum value obtained for n by plotting $\log[(V_{\mathrm{max.}}-v)/v]$ against $-\log a$ would have been 1·53. This equation is analogous to the Hill equation for the oxygenation of haemoglobin (Wyman, 1948, 1963), in which n is taken as a measure of the co-operative effects present in the haemoglobin molecule.

A further property of the enzyme system considered above is that, if now a were to be held constant while b was increased, the plot of v against b would be of different form. (This is equivalent to the situation where the values of k_{+1} and k_{+2}, k_{-1} and k_{-2}, k_{+3} and k_{+4}, k_{-3} and k_{-4} have all been interchanged, and b is held constant and v plotted against a as before.) The new relationships are shown in Table 2.

In every case, except for $a=10^{-7}$, the relationships satisfy condition (iia) and we should expect both a point of inflexion and a maximum in the plot of v against b. This is shown in Fig. 2(*b*) for $a=10^{-3}$.

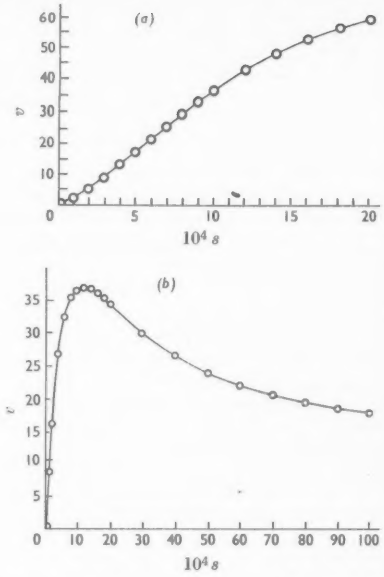


Fig. 2. Initial rate plots for a hypothetical two-substrate enzyme where the rate constants for various steps (see the text) are such as to give a kinetically preferred pathway:

$$\mathrm{E} \xrightarrow{\mathrm{A}} \mathrm{EA} \xrightarrow{\mathrm{B}} \mathrm{EXY} \longrightarrow \text{products}$$

(*a*) Constant concentration of substrate B$=10^{-3}$; concentration of substrate A increasing (i.e. $s=a$.)
(*b*) Constant concentration of A$=10^{-3}$; concentration of B increasing (i.e. $s=b$).

Table 2. *Variation in the values of the quantities involved in conditions* (i) *and* (ii) (*see the text*) *with different values of* a, *for the hypothetical enzyme described in the text*

a	$k'i'$	$m'j'$	$i'm'$	$j'l'$
10^{-2}	$1{\cdot}00\times10^{28}$	$1{\cdot}00\times10^{29}$	$1{\cdot}00\times10^{30}$	$1{\cdot}00\times10^{31}$
10^{-3}	$1{\cdot}00\times10^{25}$	$1{\cdot}00\times10^{26}$	$1{\cdot}00\times10^{28}$	$1{\cdot}00\times10^{29}$
10^{-4}	$1{\cdot}02\times10^{22}$	$1{\cdot}03\times10^{23}$	$1{\cdot}02\times10^{26}$	$1{\cdot}01\times10^{27}$
10^{-5}	$1{\cdot}21\times10^{19}$	$1{\cdot}33\times10^{20}$	$1{\cdot}20\times10^{24}$	$1{\cdot}11\times10^{25}$
10^{-6}	$4{\cdot}00\times10^{16}$	$4{\cdot}24\times10^{17}$	$2{\cdot}02\times10^{22}$	$2{\cdot}10\times10^{23}$
10^{-7}	$1{\cdot}20\times10^{15}$	$2{\cdot}40\times10^{15}$	$2{\cdot}00\times12^{21}$	$1{\cdot}20\times10^{21}$

If the initial rate eqn. (2) is recast in the form:

$$\frac{1}{v} = \frac{la^2+ma+k}{ia^2+ja} = \frac{l+m/a+k/a^2}{i+j/a} \qquad (6)$$

and if $1/v=y$ and $1/a=x$, then:

$$y = \frac{l+mx+kx^2}{i+jx} \tag{7}$$

$$\frac{dy}{dx} = \frac{kjx^2+2kix+(im-jl)}{(i+jx)^2} \tag{8}$$

$$\frac{d^2y}{dx^2} = \frac{2[ki^2-j(im-jl]}{(i+jx)^3} \tag{9}$$

These equations show that when $1/v$ is plotted against $1/a$ the curve intercepts the $1/v$ axis at l/i with a slope of $(im-jl)/i^2$. As $1/a$ approaches ∞, the slope approaches k/j. Further, from eqn. (8) there will be a minimum when:

$$\frac{1}{a} = -\frac{ki+\sqrt{k^2i^2-kj(im-jl)}}{kj}$$

If $im<jl$ this will be at a positive value of $1/a$, and the condition is the same as for a maximum in the plot of v against a, i.e. condition (ii). When condition (i) applies the reciprocal plot will have its concave side uppermost, i.e. the slope increases as $1/a$ increases, but there will be no minimum, maximum or point of inflexion. For a reciprocal plot to have its convex side uppermost, the condition is that $im>jl$ and $ki<mj$, which is also necessary for a linear reciprocal plot. In the latter case eqn. (9) shows that, in addition, $ki^2=j(im-jl)$, which may be rearranged to give $j^2l=i(mj-ki)$. In this special case the slope will be k/j.

A similar treatment shows that for the plot of a/v against a the intercept on the a/v axis will be k/j, and there will be a minimum when $ki>mj$, which is part of the condition for a sigmoid plot of v against a. As before, the plot will be linear when $ki^2=j(im-jl)$ and under these circumstances the slope will be l/i.

The most interesting of the customary methods of plotting kinetic data is the one where v is plotted against v/a. In this case the curve intercepts the v axis at i/l with a slope of $(jl-im)/il$, and the v/a axis at j/k with a slope of $kj/(ki-mj)$. When $im<jl$ there will be a maximum (as in the plot of v against a), but if $ki>mj$ the curve will double back on itself. The data for Fig. 2 are replotted in Fig. 3 in this manner, and case (a), which gave a sigmoid curve in Fig. 2, is bow-shaped in Fig. 3. Once again, if $ki^2=j(im-jl)$ the plot will be linear, with slope $-ki/jl$.

A qualitative understanding of the properties of the 'preferred order' system shown in Figs. 2 and 3 can be arrived at as follows. In case (a) of Fig. 2, that substrate, B, is held at a constant concentration which binds less readily to the free enzyme. As the concentration of the other substrate, A, is increased from very low values, at first most of it will react with EB, which will exist at a relatively high concentration, and the route followed will be almost entirely:

$$E \xrightarrow{B} EB \xrightarrow{A} EXY \longrightarrow \text{products}$$

At higher concentrations of A, however, the other, kinetically preferred, route can begin to take over, thus accounting for the upward lift in the rate curve. At very high concentrations of A the enzyme becomes saturated with A and the rate will level off. This is shown in Table 3.

In the second case the situation is reversed and the substrate, A, that is held at a constant concentration binds more readily to the free enzyme. But once the concentration of B becomes high enough to overcome the kinetic factor favouring the formation of EA more and more of the slower-reacting EB will be formed, thus reducing the overall velocity observed.

It is tempting to suppose that any steady-state system where there is a kinetically preferred pathway would give rise to the kind of sigmoid plot shown in Fig. 2(a) when the substrate, B, that is held at a constant concentration is the second one that is added on in the preferred pathway. Also, it could be supposed that holding the concentration of A constant in such a system would always give the type of curve shown in Fig. 2(b). Such suppositions can hardly be justified in view of the complexity of the conditions (i) and (ii) that govern the appearance of such curves. It is, however, reasonable to suppose that if one starts by considering the scheme of eqn. (1), with the concentration of B held constant, and chooses rate

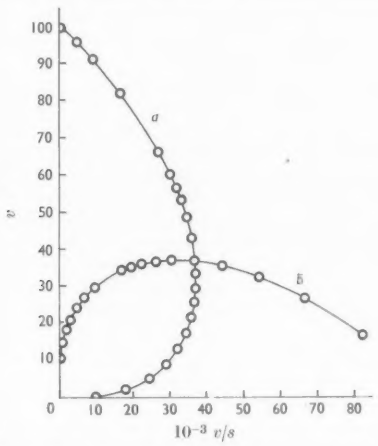


Fig. 3. Data from Fig. 2 replotted as v against v/s.

Table 3. *Distribution of enzyme among the different intermediates, and the relative flow through the two pathways shown in eqn.* 1, *for the hypothetical enzyme whose rate curve is shown in Fig.* 2(*a*)

The relative concentrations of the intermediates were calculated by solving the simultaneous steady-state equations for the hypothetical enzyme. Relative rates of flow through the two pathways were calculated by comparing the net rates of formation of EA and EB from E. Line 3 shows the situation at the point of inflexion of Fig. 2(*a*); here EA:EB=rate via EB:rate via EA=4·6.

	Percentage of total enzyme present				Percentage of total rate proceeding	
a	As E	As EA	As EB	As EXY	Via EA	Via EB
10^{-5}	1·09	0·01	98·91	0·000	9·9	90·1
10^{-4}	9·09	0·91	90·00	0·002	50·0	50·0
$4·6\times10^{-4}$	27·60	12·80	59·80	0·011	82·2	17·8
10^{-3}	33·34	33·31	33·31	0·020	91·6	8·4
10^{-2}	9·01	90·00	0·90	0·089	99·0	1·0
10^{-1}	0·99	98·90	0·01	0·100	99·9	0·1

constants that make the uppermost pathway obligatory, and then gradually changes the rate constants so as to allow the lower pathway to become possible, a point will be reached when a sigmoid plot of v against a will appear. At some later point in this process of shifting the emphasis, as the lower pathway begins to approach parity with the upper one, the condition will disappear. Later, when the lower path has assumed the greater kinetic importance a maximum will appear in the plot, finally disappearing as the lower pathway approaches the condition of being obligatory. If the whole process were now to be repeated with the concentration of A held constant, a maximum would be expected to appear first, then to disappear and be followed by a sigmoid curve that would finally disappear. The appearance of a maximum with the concentration of A held constant would probably not coincide with the appearance of a sigmoid curve when the concentration of B was held constant, because the conditions for a sigmoid curve are more stringent that those for a maximum. Presumably there is a whole range of ways of conducting the gradual transference of kinetic preference from one path to the other.

A previous investigation of limiting cases for the general rate equation (King, 1956) showed that 'unusual' kinetic consequences arise when most of the enzyme is present in the form of an intermediate that does not lie on the pathway by which most of the product is being formed. The term 'unusual' was used there to describe cases where the order of the rate equation fails to indicate the composition of the activated complex. It appears very likely that 'unusual' situations exist at or near the points of inflexion or maxima in the kinetic situations described above (see Table 3).

In work on aspartate transcarbamoylase, the elegant experiments of Gerhart & Pardee (1964), who used maleate as a competitor for aspartate, suggest very strongly that here sub-unit interactions are responsible for the sigmoid rate curves. Further, the sigmoid curve obtained for this enzyme is a fairly symmetrical one, whereas in the 'preferred pathway' cases so far examined the sigmoid curves have all been unsymmetrical with the final plateau reached only at concentrations of A much higher than those present in the region of the point of inflexion.

The kinetic data for phosphofructokinase (Atkinson & Walton, 1965; Underwood & Newsholme, 1965) are very similar to the curves shown in Fig. 2, and it may well be that the mechanism of this enzyme action involves a kinetically preferred pathway. It is true that the effects of modifiers such as AMP would require an 'effector' site on the enzyme molecule somewhere other than the active site, as suggested by Atkinson & Walton (1965). It may, however, be unnecessary to postulate other binding sites for the substrates, which would simplify the picture of the phosphofructokinase molecule considerably.

Other enzymes that show similar kinetic properties and whose mechanisms could perhaps be explained on the basis described above are NAD-isocitrate dehydrogenase of yeast (Hathaway & Atkinson, 1963) and heart malate dehydrogenase (Wolfe & Neilands, 1956; Davies & Kun, 1957). Many other enzymes that show the phenomenon of substrate inhibition when one substrate concentration is varied and the other held constant may also be of this type, even though they do not show sigmoid curves when the substrates are interchanged.

Kinetic preference may arise from effects other than conformational changes in the enzyme molecule when it combines with substrates. Further, in the case considered (Figs. 2*a* and 2*b*) the kinetic preference was unaccompanied by thermodynamic preference for any of the steps in the mechanism.

Such a situation might arise in Fig. 2(*a*) if the substrate A was a small molecule of positive charge binding to a rigid, negatively charged, active site, whereas B was a large negatively charged molecule binding at an uncharged part of the rigid active site. Other situations can be imagined in which conformational changes attendant on the binding of one substrate would affect the binding of the other in the manner described by the 'induced fit' hypothesis (e.g. Koshland, 1963). The latter situation would bear a close resemblance to the interaction postulated for haemoglobin and aspartate transcarbamoylase, except for the relatively minor difference that the interaction would be 'monosteric' rather than allosteric.

This work was carried out during the tenure of a N.A.T.O. Research Fellowship. The author acknowledges many helpful discussions with Dr S. Ainsworth and Professor W. Bartley.

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The Uncoupling of Respiratory-Chain Phosphorylation by 4,5,6,7-Tetrachloro-2-trifluoromethylbenzimidazole

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(*Received* 25 *June* 1965)

1. 30mμM-4,5,6,7-Tetrachloro-2-trifluoromethylbenzimidazole (TTFB) uncouples respiratory-chain phosphorylation. The respiration rate of uncoupled mitochondria is significantly greater than the state 3 rate. Neither added orthophosphate nor ATP is required to sustain this rate. The oligomycin- and octylguanidine-induced inhibitions of respiration are relieved by uncoupling concentrations of TTFB. 2. Uncoupling concentrations of TTFB elicit a high adenosinetriphosphatase activity and inhibit the ATP-dependent succinate-linked reduction of NAD catalysed by submitochondrial particles from ox-heart mitochondria. 3. The action of TTFB is similar to that of 2,4-dinitrophenol. Evidence is presented which shows that it is the anionic forms of benzimidazoles and imidazoles, having the negative charge localized on the ring nitrogen atom, which are the effective uncoupling agents.

The biological activity of substituted benzimidazoles has been studied in some detail. It has been reported that benzimidazoles inhibit the growth of many organisms, e.g. *Saccharomyces cerevisiae* (Wooley, 1944), various bacteria (Hendlin & Soars, 1951; Greer, 1958; McNair-Scott, Rogers & Rose, 1958; Bishop, Chelton & Jones, 1964), three viruses (Thompson, 1947; Brown, 1952; Tamm, Folkers, Shunk, Heyl & Horsfall, 1953), protozoans (Gillespie, Engelman & Graff, 1954; Timmis & Epstein, 1959) and amphibian embryos (Gillespie *et al.* 1954). The mode of action has been ascribed variously to the blocking of purine utilization and interference with the metabolism of vitamin B_{12}, which contains the 5,6-dimethyl-benzimidazole moiety. Benzimidazole itself produces a variety of pharmacological responses in mammalian tissues and organs (for references see McNair-Scott *et al.* 1958).

The work in the present paper shows that TTFB* is a potent uncoupler of respiratory-chain phosphorylation. The mechanism and locus of action of TTFB are apparently identical with those of 2,4-dinitrophenol. Evidence is presented which suggests that it is the anion of TTFB which is the active uncoupling agent.

A preliminary report of this work has appeared (Büchel, Korte & Beechey, 1965).

* Abbreviation: TTFB, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole.

METHODS

Preparation of mitochondria and submitochondrial particles. Rat-heart mitochondria were prepared by the method of Chance & Hagihara (1961). Ox-heart mitochondria were prepared by the method of Sanadi & Fluharty (1963). Submitochondrial particles (ETP_H) were prepared by the method of Hansen & Smith (1964).

Assay of protein. Protein was estimated colorimetrically by the biuret method of Gornall, Bardawill & David (1949).

Estimation of respiration rates. A Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A.) was used to measure oxygen concentrations in an apparatus similar to that described by Chappell (1964*a*). The temperature of the apparatus was maintained at 25°. Additions to the reaction vessel were made with microsyringes. The pH of all the aqueous solutions used in the reaction vessel was adjusted to 7·4. The oxygen concentration of the air-saturated reaction media was taken as 237μM (Chappell, 1964*a*).

Manometric assay of P/O *ratios.* Rat-heart mitochondria were suspended in a reaction medium containing succinate (5mM), $MgCl_2$ (5mM), ATP (1mM), KH_2PO_4–Na_2HPO_4 buffer, pH 7·4 (20mM), and sucrose (0·15M). A 0·9ml. sample of this suspension (3–4mg. of protein) was placed in the main compartment of a 5ml. respirometer flask, with 0·1ml. of 20% (w/v) KOH in the centre well and 0·1ml. of 0·25M-glucose containing 1mg. of crystalline hexokinase (Sigma type III) in the side arm. After a 10min. equilibration period at 25° the contents of the side arm and main compartment were mixed and the oxygen consumption was measured. The reaction was stopped by the addition of 0·1ml. of 40% (w/v) trichloroacetic acid. The uptake of

orthophosphate was determined as the difference between the orthophosphate content of the experimental flasks and zero-time controls to which glucose–hexokinase solution was added after the addition of trichloroacetic acid. Orthophosphate was assayed by the method of Sumner (1940).

Reversed-phase thin-layer chromatography. A slurry of 13g. of silica gel HF_{254} (Merck) and 16g. of Avicel (technical-grade microcrystalline cellulose; Avicel Sales, Marcus Hook, Pa. 19061, U.S.A.) in 83ml. of water was spread over thin-layer plates to a thickness of 250μ. After drying at room temperature the plates were impregnated with liquid paraffin by allowing a 10% (v/v) solution of liquid paraffin B.P. in hexane to ascend the plate and then evaporating off the hexane in a stream of air. After application of the substances to be separated the chromatogram was developed in water–acetone (4:1, v/v). The substances were located by inspection under monochromatic light (254mμ). This method is rapid and very convenient for compounds that are not otherwise easily detectable (B. Bush, personal communication).

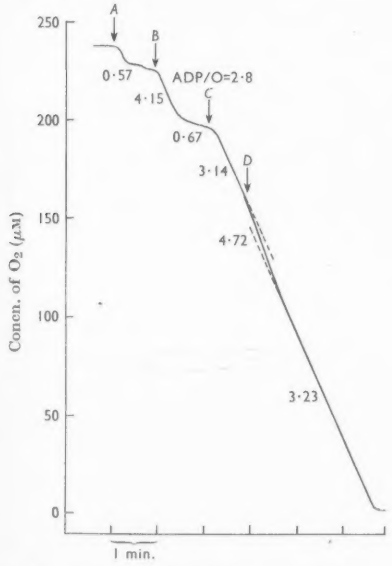

Fig. 1. Uncoupling action of TTFB on the coupled respiration of rat-heart mitochondria. The line represents the output from an oxygen electrode. The temperature was 25°. At *A*, 50μl. of heart mitochondria (2·1mg. of protein), suspended in 0·25M-sucrose–mM-EDTA, was added to a reaction mixture containing 3·5ml. of 0·25M-sucrose–mM-EDTA, 50μl. of M-potassium phosphate buffer, pH7·4, and 50μl. of M-potassium glutamate. At *B*, 10μl. of 0·04M-ADP (potassium salt) was added. At *C*, 5μl. of 10μM-TTFB in ethanol was added, giving a final concentration of 13·5mμM. At *D*, a further 10μl. of 0·04M-ADP (potassium salt) was added. The values on the curve represent the respiration rate expressed in μmoles of oxygen/mg. of protein/hr.

RESULTS

Effects of TTFB on the coupled respiration of rat-heart mitochondria. (*a*) With orthophosphate and ATP present. Fig. 1 is an oxygen-electrode trace of an experiment in which mitochondria were added to an aerated reaction medium containing glutamate and orthophosphate buffer, pH7·4. ADP (0·4μmole) was then added and the mitochondrial respiration rate showed a typical state 4,3,4 cycle, the ADP/O ratio being 2·8. The ADP/O ratio is calculated on the assumption that the oxygen consumption during the rapid phase of oxygen consumption after the addition of ADP, state 3, is associated with the phosphorylation of ADP to ATP (Chance & Williams, 1955). On the addition of TTFB (final concn. 11mμM), the respiration rate immediately changed to an increased and constant rate. A second addition of ADP again stimulated the respiration to a rate 14% greater than the state 3 respiration rate measured previously. The respiration rate eventually declined to a rate similar to that measured before the second addition of ADP. The ADP/O ratio for this state 4,3,4 cycle in the presence of TTFB was 1·1.

This type of experiment was repeated to establish the minimum concentration of TTFB that prevents the stimulation of respiration by a subsequent addition of ADP. This concentration of TTFB (30mμM) was designated the 'minimum uncoupling concentration'. As the concentration of TTFB was increased still further the stimulation of respiration declined and an inhibition of respiration occurred. This inhibition was not relieved by ADP.

P/O ratios were also estimated by using manometers to assay the oxygen consumption and a hexokinase–glucose trap to enable ATP synthesis to be measured by the orthophosphate-uptake method. Under these conditions 30mμM-TTFB completely inhibited the synthesis of ATP.

(*b*) With ATP absent. The stimulation of respiration by TTFB was not dependent on the prior addition of ADP and its subsequent phosphorylation to ATP. The addition of an uncoupling concentration of TTFB to mitochondria in state 4 caused a stimulation of the respiration to a rate greater than the state 3 rate measured in control experiments.

(*c*) With orthophosphate and ATP absent. In these experiments mitochondria were added to an aerated reaction medium similar to that described in Fig. 1, save that 2·5mM-tris–chloride buffer, pH7·4, was substituted for orthophosphate buffer. The addition of an uncoupling concentration of TTFB stimulated the respiration to a rate which was 90% of that of the state 3 rate measured in control experiments. A similar result was obtained with α-oxoglutarate and succinate as substrates.

Reversal of the uncoupling action of TTFB by

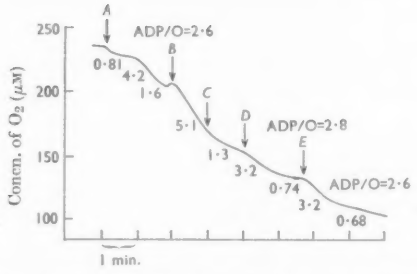

Fig. 2. Reversal of TTFB uncoupling by bovine plasma albumin. The line represents the output from an oxygen electrode. The temperature was 25°. At *A*, mitochondria (1·1mg. of protein), suspended in 0·1ml. of 0·25M-sucrose–mM-EDTA, were added to a reaction mixture containing 4·1ml. of 0·25M-sucrose–mM-EDTA, 10μl. of M-potassium phosphate buffer, pH7·4, and 20μl. of M-potassium glutamate. At *B*, 10μl. of 10μM-TTFB in ethanol was added, giving a final concentration of 24mμM. At *C*, 2·5mg. of crystalline bovine plasma albumin in 50μl. of water was added. At *D* and again at *E*, 10μl. of 0·04mM-ADP (potassium salt) was added. The values on the curve represent the respiration rate expressed in μmoles of oxygen/mg. of protein/hr.

bovine plasma albumin. The oxygen-electrode trace illustrated in Fig. 2 shows rates of oxygen consumption of heart mitochondria respiring in a reaction medium containing glutamate and orthophosphate. The addition of ADP causes a state 4,3,4 cycle. The respiration rate increases on the addition of TTFB (final concn. 24mμM) to a rate that is 18% greater than the state 3 rate. The slight increase in the electrode output is an anomaly caused by the addition of ethanol. Within 1min. after the addition of 2·5mg. of crystalline bovine plasma albumin the respiration rate returned to a state 4 rate. Subsequent additions of ADP stimulated typical state 4,3,4 cycles with normal P/O ratios but somewhat lower state 3 respiration rates.

Relief by TTFB of respiratory inhibition caused by oligomycin and octylguanidine. The experiment illustrated in Fig. 3(*a*) shows the stimulation of respiration by TTFB in an oligomycin-inhibited mitochondrial preparation. The mitochondria were respiring in a reaction medium containing orthophosphate, glutamate and malate as substrates with malonate to inhibit the oxidation of succinate. On the addition of ADP the respiration rates showed a typical state 4,3,4 cycle. The addition of 6μg. of oligomycin A had no effect on the state 4 respiration rate, but prevented the onset of a state 3 respiration rate when a second addition of ADP was made. However, the addition of TTFB (final concn. 140mμM) caused an immediate stimulation

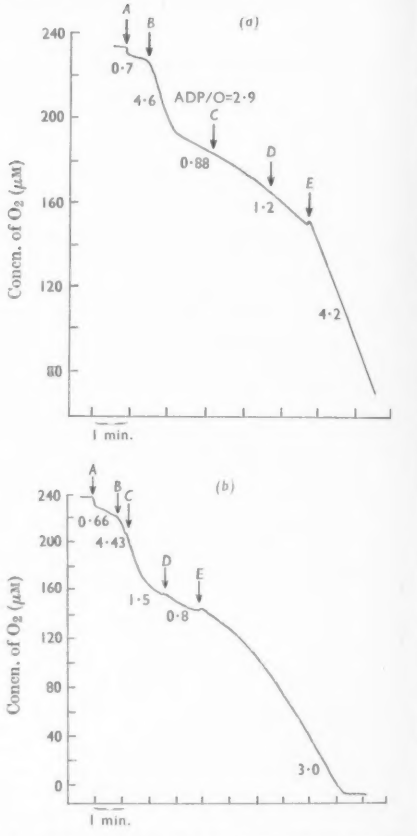

Fig. 3. Relief by TTFB of the oligomycin (*a*) and octylguanidine (*b*) inhibitions of coupled respiration. The lines represent the output from an oxygen electrode. The temperature was 25°. At *A*, mitochondria (1·6mg. of protein), suspended in 0·1ml. of 0·25M-sucrose–mM-EDTA, were added to a reaction mixture containing 4·1ml. of 0·25M-sucrose–mM-EDTA, 50μl. of M-potassium phosphate buffer, pH7·4 and 40μl. of a substrate solution containing potassium malonate (0·5M), potassium malate (0·25mM) and potassium glutamate (0·25M). At *B*, 25μl. of 0·04M-ADP (potassium salt) was added. At *C*, 6μg. of oligomycin A in 6μl. of ethanol (*a*) or 20μl. of 20mM-octylguanidine in ethanol (*b*) was added. At *D*, a further 25μl. of 0·04M-ADP (potassium salt) was added. At *E*, 6μl. of 100μM-TTFB in ethanol was added.

of the respiration to a rate that was 90% of the previous state 3 rate.

A similar reaction medium was used in the experiment illustrated in Fig. 3(*b*). In this experiment octylguanidine (final concn. 125μM) was added

$n = 0, 1, 2, 3, 4$

Scheme 1.

while the mitochondria were in state 3. The respiration rate declined to a state 4 rate but the mitochondria did not respond to a further addition of ADP. When TTFB (final concn. 270mμM) was added the respiration rate slowly accelerated to a linear respiration rate that was 68% of the previous state 3 rate.

Effect of TTFB on mitochondrial adenosine-triphosphatase activity. The data listed in Table 1 show that, as the concentration of TTFB in the adenosine-triphosphatase assay was increased to 24mμM, the adenosine-triphosphatase activity increased to a maximum, which was 27% higher than the adenosine-triphosphatase activity stimulated by an uncoupling concentration of dinitrophenol (30μM). The adenosine-triphosphatase activity fell slightly when the concentration of TTFB in the adenosine-triphosphatase assay was increased to 300mμM.

Effect of TTFB on the ATP-dependent succinate-linked reduction of NAD. Submitochondrial particles were prepared from ox-heart mitochondria; they catalysed the ATP-dependent succinate-linked reduction of NAD at a rate of 50mμmoles/mg. of protein/min. at 30° when assayed under the conditions described by Sanadi & Fluharty (1963). 80mμM-TTFB caused an 86% decrease in this rate. Control experiments showed that small additions of ethanol had no effect on this reaction.

Variation of uncoupling activity with the pK_1 *of the substituted benzimidazole.* The uncoupling concentrations of 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole, 4,5,6-trichloro-2-trifluoromethylbenzimidazole, 5,6-dichloro-2-trifluoromethylbenzimidazole, 5-chloro-2-trifluoromethylbenzimidazole, 5-nitro-2-trifluoromethylbenzimidazole and 2-trifluoromethylbenzimidazole were assayed as described above. Apparent pK_1 values for the dissociation shown in Scheme 1 were measured by titration of solutions of these compounds in 50% (v/v) ethanol with aq. potassium hydroxide solutions.

The plot of apparent pK_1 values against the log (minimum uncoupling concentration) is shown in Fig. 4. As the number of chlorine atoms substituted in the benzimidazole nucleus increases, the value of the apparent pK_1 falls and this decrease is accompanied by an approximately logarithmic increase in the uncoupling activity of the molecules. 5-Nitro-2-trifluoromethylbenzimidazole has pK_1

Table 1. *Effect of 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole on the adenosine-triphosphatase activity of rat-heart mitochondria*

Mitochondria (1·1mg. of protein) suspended in 0·2ml. of 0·25M-sucrose–mM-EDTA, pH7·4, were added at zero time to 0·8ml. of a reaction mixture containing sucrose (0·125M), tris–chloride buffer, pH7·4 (63mM), ATP (2·5mM) and either 10μl. of an ethanolic solution of TTFB or an aqueous solution of dinitrophenol (3mM). The reaction was stopped after 15min. at 20° by the addition of 0·1ml. of 40% (w/v) trichloroacetic acid. The orthophosphate content of the supernatant was assayed by the method of Sumner (1944). Zero-time, enzyme and substrate controls were included. The data presented have been corrected for these control assays, which were less than 4% of the maximum activity shown here.

Uncoupler	Concn. (mμM)	Adenosine-triphosphatase activity (μmoles/hr./mg. of protein)
TTFB	3	1·3
	6	2·6
	12	4·9
	24	6·6
	30	6·5
	150	6·4
	300	6·0
Dinitrophenol	30000	5·2

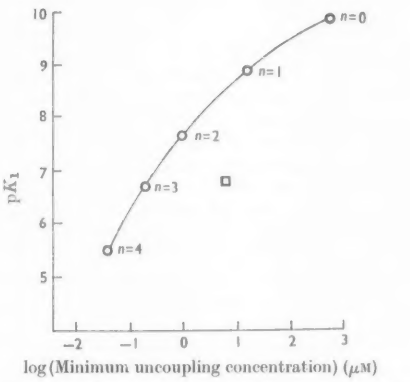

Fig. 4. Relationship between the minimum uncoupling activities and the pK_1 of substituted 2-trifluoromethylbenzimidazoles. ○, *n*-Chloro-2-trifluoromethylbenzimidazoles (*n* is the number of chlorine substituents); □, 5-nitro-2-trifluoromethylbenzimidazole.

6·7 and its measured minimum uncoupling concentration was 5μM. Thus the data for this particular compound do not fit the relationship obtained for the chlorine-substituted 2-trifluoromethylbenzimidazoles.

4,5,6-Trichloro-*N*-methyl-2-trifluoromethylbenzimidazole at concentrations up to 75μM was completely devoid of uncoupling activity.

DISCUSSION

The results presented here show that TTFB is a potent uncoupler of respiratory-chain phosphorylation and that its mode of action appears to be identical with that of 2,4-dinitrophenol. Thus TTFB relieves the oligomycin-induced inhibition of respiration (cf. Lardy, Johnson & McMurray, 1958); TTFB also relieves the octylguanidine-induced inhibition of respiration (cf. Pressman, 1963); TTFB increases the respiration rate of mitochondria in state 4 to a rate that is significantly greater than the state 3 rate (cf. Chappell, 1964*b*); TTFB induces adenosine-triphosphatase activity in mitochondria (cf. Hunter, 1951); the uncoupling action of TTFB is reversed by bovine plasma albumin (cf. Garbus & Weinbach, 1963); uncoupling concentrations of TTFB also inhibit the ATP-dependent reduction of NAD by succinate (cf. Löw, Krueger & Ziegler, 1961). However, TTFB uncouples at 30mμM and hence is about 1000-fold as potent as dinitrophenol.

In the absence of orthophosphate, the stimulation of succinate oxidation by TTFB is similar to the action of dinitrophenol. However, the stimulation of α-oxoglutarate and glutamate oxidations by TTFB in absence of orthophosphate contrasts with the inertness of dinitrophenol under similar conditions (Borst & Slater, 1961).

The data illustrated in Fig. 4 show that the lower the pK_1 of the substituted benzimidazole (i.e. the greater the degree of dissociation of the compound at pH 7·4) the more efficient it is as an uncoupling agent. Thus it appears that the active uncoupler is the substituted benzimidazole anion. This conclusion is supported further by the facts that at pH 7·4 TTFB is 99·7% ionized and that a compound which cannot undergo the dissociation shown in Scheme 1, i.e. 4,5,6-trichloro-*N*-methyl-2-trifluoromethylbenzimidazole, is completely without uncoupling activity. This theory is backed by additional results obtained with substituted imidazoles. The data in Table 2 show that 2,4,5-tribromoimidazole (pK_1 6·95) is a more efficient uncoupler than 2,4-dibromoimidazole (pK_1 9·8) and also that 2,4,5-tribromo-*N*-methylimidazole is devoid of uncoupling activity.

Parker (1958) concluded that the dissociated forms of substituted nitro- and halogeno-phenols were the actual uncoupling agents. However,

Table 2. *Effect of substituted imidazoles on the coupled respiration of rat-heart sarcosomes*

The uncoupling activity was estimated by the method described in the text.

	pK_1	Minimum uncoupling concentration
2,4,5-Tribromoimidazole	6·95	13μM
4,5-Dibromoimidazole	9·8	740μM
2,4,5-Tribromo-*N*-methyl imidazole	No dissociation	No uncoupling at 1·35mM
2,4-Dibromo-5-nitro-imidazole	3·6	P/O ratio decreased by 20% at 700μM
4,5-Dicyanoimidazole	5·05	P/O ratio decreased by 62% at 950μM

Hemker (1962) and Gladtker & Liss (1959) have suggested that the uncoupling activities of 4-alkyl-2,6-dinitrophenols are directly determined by the amount of undissociated phenol dissolved in the mitochondrial lipid. The variation of the uncoupling activity shown in Fig. 4 cannot be significantly ascribed to the expected increase in lipid solubility as the number of chlorine substituents is increased, since reversed-phase thin-layer partition chromatography of these compounds showed the ratio of the maximum R_F to minimum R_F to be 4, whereas the ratio of the uncoupling activities is 10^4. The undissociated form of TTFB is highly lipophilic; in contrast, the anionic form is readily soluble in water at pH 7·4 and at the concentrations used in these experiments. It is envisaged that TTFB penetrates the mitochondrial membrane in the undissociated form and assumes its dissociated form when within the mitochondria. In this form it can then penetrate the polar active site of the enzyme involved. Weinbach & Garbus (1964) have proposed a similar mechanism for the penetration and mode of action of halogeno- and nitro-phenols.

Table 2 and Fig. 4 show that the benzimidazoles and imidazoles substituted with electrophilic nitro or cyano groups do not have the uncoupling activity which might be predicted from a comparison of their measured pK_1 values with those of the halogenated benzimidazoles and imidazoles. In these molecules it is highly probable that the mesomeric effect induced by the nitro and cyano groups will delocalize the negative charge produced by the dissociation of the ring –NH– group. Thus, in addition to the dissociation of the benzimidazoles and imidazoles, the localization of the negative charge on the ring nitrogen atom is a prerequisite for an effective uncoupling agent. The minimum uncoupling concentration of 5-nitro-2-trifluoromethylbenzimidazole, namely 6μM, is 30 times that of 4,5,6-trichloro-2-trifluoromethylbenzimidazole, a compound with a similar pK_1. Also, 2,4-dibromo-5-nitroimidazole is at least 2000-fold less effective than would be expected from its pK_1. (This esti-

mate is based on the data listed in Table 2.) Since the mesomeric delocalization of the negative charge would be expected to be similar in both molecules it appears that the nitro group in 2,4-dibromo-5-nitroimidazole is causing a neighbouring-group effect in addition to the delocalization of the negative charge.

Imidazole at very high concentrations (25mM) has been shown to inhibit the ATP-dependent succinate-linked reduction of NAD (Hommes, Estabrook, Chance & Rasmussan, 1964) and to uncouple partially oxidative phosphorylation (Conover, Gonze & Estabrook, 1964) in submitochondrial particles from ox heart. Both groups of workers concluded that 'imidazole may act by competing in the energy-transfer reaction between a phosphorylated high-energy intermediate and its energy acceptor'. The present results suggest that imidazole is acting in a manner similar to dinitrophenol and hence at a site prior to the involvement of a phosphorylated intermediate (Borst & Slater, 1961; Chappell, 1964*b*).

With the exception of some of the benzimidazoles used by Bishop *et al.* (1964), all of the substituted benzimidazoles used in the experiments quoted in the introduction will have pK_1 values that are too high for these compounds to be active as uncoupling agents at the concentrations used. However, Bishop *et al.* (1964) state that the mode of action of 4,5-bistrifluoromethylbenzimidazole was not as an antimetabolite of vitamin B_{12}, guanine, adenine or histidine. In this case, and for other fluorine-substituted benzimidazoles quoted by these workers, the bactericidal activity of these compounds is probably due to the uncoupling of respiratory-chain phosphorylation.

I am indebted to Mr I. G. Knight for skilled technical assistance. I thank Dr K. H. Büchel, Dr H. Röchling and Dr W. Draber of Shell Grundlagenforschung-G.m.b.H., who synthesized the benzimidazoles and imidazoles used in these experiments and who also provided some of the pK_1 values used. My thanks also are due to Professor Van Tamelen for a gift of oligomycin A, Mr B. Wallace for synthesizing the octylguanidine, Dr B. Bush for advice on thin-layer chromatography and Dr R. P. M. Bond for much valuable discussion. The constructive criticism and help given by Lord Rothschild, F.R.S., Dr G. J. Popják, F.R.S., and Dr J. W. Cornforth, F.R.S., was greatly appreciated.

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Dextran-Gel Filtration of Enzymes in the Presence of their High-Molecular-Weight Substrates

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(*Received* 15 *July* 1965)

1. Trypsin and ribonuclease were filtered through dextran gel (Sephadex G-100) columns in the absence and presence of their respective substrates. In the presence of their high-molecular-weight substrates the enzymes emerged earlier from the columns. This appeared to be due to the reversible formation of specific enzyme–substrate complexes. 2. The possibility of separation of an enzyme from other proteins with similar molecular weights was demonstrated with trypsin and cytochrome *c* in the presence of casein.

When proteins are filtered through a gel column their elution volumes are essentially a function of their molecular weights (Andrews, 1962; Wieland, Duesberg & Determann, 1963; Whitaker, 1963; Iwatsubo & Curdel, 1963; Andrews, 1964; Auricchio & Bruni, 1964). Thus proteins with widely differing molecular weights can usually be separated, whereas proteins with similar molecular weights emerge together.

During gel filtration of a mixture of proteins, one of which is an enzyme, if the column of gel is equilibrated with a solution containing a high-molecular-weight substrate of the enzyme then it is to be expected that reversible formation of the enzyme–substrate complex will decrease the elution volume of the enzyme. A specific separation of one enzyme from a mixture of proteins of similar molecular weight might thus be achieved. The purpose of the present paper is to demonstrate that by dextran-gel filtration a protein can be separated from other molecules of similar size if it forms a complex with a high-molecular-weight substance. This method of separation has been examined with trypsin and ribonuclease.

MATERIALS AND METHODS

Cytochrome *c* (from horse heart), type II, was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.; ribonuclease, trypsin, malate dehydrogenase, lactate dehydrogenase, glutamate dehydrogenase and yeast soluble RNA were from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany; casein prepared according to Hammarsten was from E. Merck A.-G., Darmstadt, Germany; benzoyl-L-arginine ethyl ester hydrochloride was from Seravac Laboratories (Pty.) Ltd., Maidenhead, Berks. All other reagents were of analytical grade.

Sephadex G-100 (lot no. To2946; particle size 40–120μ) (Pharmacia, Uppsala, Sweden) was suspended in a solution of 6M-urea, stirred with a magnetic stirrer for about 1hr. and exhaustively washed with water. The smallest particles were removed by decantation, and then a suspension of the gel was deaerated and packed into columns (1·5cm. diam. $\times$ 79cm.). Columns of gel were equilibrated with the desired buffer, which was allowed to pass through until the height of the gel bed remained constant. Proteins were dissolved in 1ml. of the buffer used for equilibration, pipetted on to the top of the column and eluted with the same buffer. The same procedure was used with buffers containing 1% (w/v) of casein, 0·5% of soluble RNA or 0·02% of benzoyl-L-arginine ethyl ester hydrochloride.

Casein was brought into solution by boiling a 1% suspension in 35mM-phosphate buffer, pH7·6, prepared as described by Sörensen (1909), and containing NaCl (0·2M), cooling and filtering the solution, and adjusting its pH to 7·6 with 2N-NaOH. Before use a 0·5% solution of soluble RNA in 0·1M-sodium acetate buffer, pH5·2, containing NaCl (0·2M), was dialysed against the same buffer for 4 days.

The effluent volume corresponding to maximum concentration of a solute (V_e) was estimated as described by Andrews (1964) to the nearest 1ml. from an elution diagram by extrapolating both sides of the solute peak to an apex. All filtrations were done at 3°. The flow rates of the columns were approx. 10ml./hr. In the absence of substrates the concentrations of the proteins in the effluent fractions were estimated from the extinction readings at 210 or 280mμ according to the quantity of protein used. Cytochrome *c* was estimated at 408mμ. The concentrations of ribonuclease (Anfinsen, Redfield, Choate, Page & Carroll, 1954), malate dehydrogenase (Mehler, Kornberg, Grisolia & Ochoa, 1948), trypsin (Kunitz, 1947; Schwert & Takenaka, 1955) and lactate dehydrogenase (Kornberg, 1955) were determined by the appropriate enzymic test.

RESULTS

Sephadex G-100 columns were equilibrated with 35mM-phosphate buffer, pH7·6, containing sodium chloride (0·2M) and were calibrated for molecular-weight determination with cytochrome *c* (mol.wt.

13000) (Paléus & Paul, 1963), ribonuclease (mol.wt. 13700) (Hirs, Moore & Stein, 1956), trypsin (mol.wt. 23800) (Cunningham, 1954), malate dehydrogenase (mol.wt. 70000) (Thorne & Kaplan, 1963) and lactate dehydrogenase (mol.wt. 117800) (Jaenicke, 1963). A calibration curve was obtained by plotting the elution volumes of the proteins against the logarithms of their respective molecular weights (Andrews, 1964).

The elution volume of trypsin was not affected when from 0·1 to 20mg. was submitted to gel filtration. However, when the columns were equilibrated with and eluted with a 1% casein solution, the elution volumes were decreased and dependent on the amount of trypsin introduced into the column. The lower the trypsin concentration, the smaller was the elution volume (Table 1). When 0·1mg. of trypsin was introduced into the column, the elution volume corresponded to mol.wt. 100000. There was no significant difference in the elution volumes of cytochrome *c*, ribonuclease and lactate dehydrogenase when submitted to gel filtration in the presence or absence of casein (Table 1). Neither was the elution volume of trypsin changed when it was filtered on a column equilibrated with the low-molecular-weight substrate benzoyl-L-arginine ethyl ester hydrochloride.

Similar experiments were performed with ribonuclease. The Sephadex G-100 columns were equilibrated with 0·1M-sodium acetate buffer, pH5·2, containing sodium chloride (0·2M). The elution volume of 0·1mg. of ribonuclease submitted to gel filtration in the presence of dialysed 0·5% soluble RNA solution was significantly smaller than in the absence of RNA. At a higher concentration of ribonuclease (1mg.) the elution volume was not significantly different in the presence or absence of RNA. The elution volumes of malate and lactate dehydrogenase were not affected by soluble RNA, but the appearance of the cytochrome *c* from the column was accelerated (Table 2).

A mixture of cytochrome c (20mg.) and trypsin (0·1mg.) was submitted to gel filtration in the

Table 1. *Gel-filtration elution volumes of trypsin and other proteins in the absence and presence of casein*

The proteins were filtered through Sephadex G-100 columns (1·5cm. × 79cm.) equilibrated with 35mM-phosphate buffer, pH7·6, containing NaCl (0·2M), in the absence and presence of 1% (w/v) of casein.

Protein	Wt. used (mg.)	V_e in absence of casein (ml.)	V_e in presence of casein (ml.)
Cytochrome *c*	1	98	100
Ribonuclease	1	97	95
Lactate dehydrogenase	1	50	52
Trypsin	0·1	85	53
Trypsin	1	85	68
Trypsin	10	85	71
Trypsin	20	85	77
Trypsin	60	—	79

Table 2. *Gel-filtration elution volumes of ribonuclease and other proteins in the absence and presence of soluble RNA*

The proteins were filtered through Sephadex G-100 columns (1·5cm. × 79cm.) equilibrated with 0·1M-sodium acetate buffer, pH5·2, containing NaCl (0·2M), in the absence and presence of 0·5% of soluble RNA.

	Wt. used (mg.)	V_e in absence of RNA (ml.)	V_e in presence of RNA (ml.)
Cytochrome *c*	0·1	98	91
Malate dehydrogenase	0·1	61	61
Lactate dehydrogenase	0·1	50	52
Ribonuclease	0·1	96	86
Ribonuclease	1	96	94

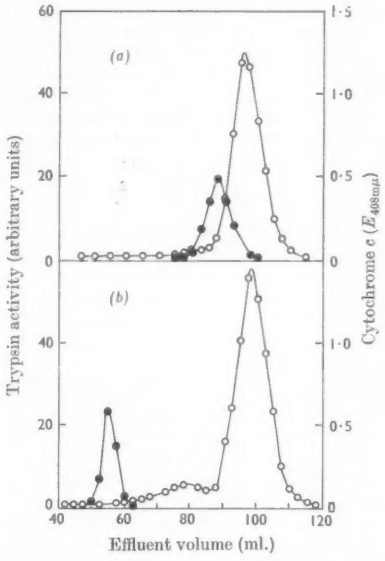

Fig. 1. Elution diagram of a mixture of 20mg. of cytochrome *c* (○) and 0·1mg. of trypsin (●) on a Sephadex G-100 column (1·5cm. × 79cm.) equilibrated with 35mM-phosphate buffer, pH7·6, containing NaCl (0·2M), in the absence (*a*) and in the presence (*b*) of 1% of casein.

presence and absence of 1% casein. In the absence of casein there was a slight separation (Fig. 1*a*), whereas in the presence of casein there was a complete separation of the proteins (Fig. 1*b*). After concentration by vacuum-dialysis at 3°, the trypsin was separated from casein by a second gel filtration in the absence of casein.

DISCUSSION

The results obtained by filtering trypsin at different concentrations through columns of dextran gel equilibrated with buffer plus casein show that, within certain limits, the elution volume of a protein can be varied. Under the conditions of our experiments the variation in the elution volume for trypsin does not seem to be due to an alteration in the mechanism of the gel-filtration process induced by the presence of casein. Proteins with lower molecular weights (cytochrome *c* and ribonuclease) and with a higher molecular weight (lactate dehydrogenase) than that of trypsin were eluted as expected from their molecular weights even in the presence of casein. It is improbable that the variation of the elution volume is due to a change in form of the enzyme in the presence of substrate, since a low-molecular-weight substrate, benzoyl-L-arginine ethyl ester hydrochloride, caused no change in the elution volume of trypsin. The possibility that casein can accelerate the elution of trypsin from the Sephadex column by neutralizing a charge complex between trypsin and gel existing in the absence of casein can also be excluded, because in the absence of casein the elution volume of trypsin was that expected on the basis of its molecular weight, whereas in the presence of casein the elution volumes corresponded to much higher molecular weights.

The concentration-dependence of the elution volume of trypsin in the presence of casein seems to be due to the reversible formation of an enzyme–substrate complex. The reversibility is clearly shown by the subsequent separation of trypsin from casein in a column equilibrated with buffer containing no casein.

The molecular weight of the substrate that binds to the enzyme is an important factor in determining the degree of reduction of the elution volume. A small substrate such as benzoyl-L-arginine ethyl ester hydrochloride does not significantly affect the elution volume of trypsin. On the other hand, the binding of the very large casein molecules greatly increases the size of the protein moiety being filtered, and causes a considerable reduction in the elution volume.

The variations in the elution volume of ribonuclease in the presence of soluble RNA were relatively small when compared with those of trypsin in the presence of casein. This, however, was expected since the elution volume of the RNA corresponded to that of a protein with mol.wt. 17000 whereas the casein emerged at the void volume of the column (it had the same elution volume as 40mg. of glutamate dehydrogenase) and therefore existed in a form with mol.wt. greater than 300000 (Andrews, 1964).

The change in the elution volume of the basic cytochrome *c* in the presence of the acidic RNA shows that complexes other than the highly specific enzyme–substrate type may also be formed and alter the normal elution volume of a protein.

The separation of trypsin from cytochrome *c* in the presence of casein illustrates a possible application of the phenomenon that we have described. In favourable cases it might be possible to purify an enzyme from a crude preparation by only two steps: an initial gel-filtration step to separate the enzyme together with other proteins of the same size from the remainder of the proteins; then a second gel-filtration step in which a specific variation in the elution volume of the enzyme is induced, for instance by the presence of high-molecular-weight substrate, to separate it from other proteins of similar size.

We thank Dr P. Andrews for his kind interest and criticism and Dr J. J. Holbrook for useful discussions and help in the preparation of the text.

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The Differentiation and Assay of Vitamins D_2 and D_3 by Gas–Liquid Chromatography

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(*Received* 27 *July* 1965)

1. A method is described for the differentiation and determination of as little as 0·2 μg. of vitamins D_2 and D_3 by gas–liquid chromatography. 2. The vitamins are converted by treatment with antimony trichloride into isovitamins D_2 and D_3, which show single, separate peaks on gas–liquid chromatography, unlike the unmodified vitamins, which give twin peaks due to the formation of pyro and isopyro derivatives. 3. Since isovitamins D_2 and D_3 remain together in all steps of the procedure except during gas–liquid chromatography, one may be used as an internal standard for the other. 4. The use of an internal standard reduces the importance of loss during sample preparation and increases precision. 5. The application of the method to biological materials is demonstrated.

Many methods have been devised for the assay of vitamin D but none has been entirely satisfactory with regard to sensitivity, specificity, precision and ability to distinguish between vitamins D_2 and D_3. Gas chromatography, which has often provided the means of assay when other methods have failed, was first investigated as a method for vitamin D by Ziffer, Vanden Heuvel, Haahti & Horning (1960) and later applied to the assay of rat liver by Nair, Bucana, de Leon & Turner (1965). Vitamins D_2 and D_3 are converted, at the temperature of vaporization, to the 'pyro' and 'isopyro' forms and appear as twin peaks in gas chromatograms. Nair *et al.* (1965) demonstrated that these derivatives are formed in constant proportion and that they can be separated by gas chromatography. An examination of the relative retention volumes of the four derivatives indicates that the peaks fall near to each other and, were they present together, would be difficult to distinguish and measure. Cholesterol emerges between the pyro and isopyro forms and it is essential that it be eliminated before gas chromatography.

The present paper describes a sensitive and relatively simple method for the assay of vitamins D_2 and D_3 in which the vitamins are modified to give single, separate peaks during gas chromatography and in which one is used as an internal standard in the assay of the other. For preliminary communication, see Murray, Day & Kodicek (1965).

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EXPERIMENTAL

Materials. Crystalline vitamin D_2 (Glaxo Laboratories Ltd., Greenford, Middlesex) and vitamin D_3 (Koch–Light Laboratories Ltd., Colnbrook, Bucks.) were dissolved in light petroleum (b.p. 40–60°) as required for standards or in arachis oil for animal dosing. The light petroleum was shaken twice with conc. H_2SO_4, washed with water until acid-free and redistilled. Ethanol was redistilled; other solvents and chemicals (analytical grade) were used as received.

Animal experiments. Weanling, hooded rats were given the rachitogenic diet 2965 of Steenbock & Black (1925) modified by the addition of 0·05% of lysine and the following B-vitamins (mg./kg.): thiamine 2, riboflavine 4, niacin 10, calcium pantothenate 4. Each rat received 7g. of fresh carrot/week. After at least 3 weeks they were dosed orally with 250 or 500 μg. of vitamin D_2 or D_3 in arachis oil and were decapitated at various time-intervals. Undosed rachitic rats were killed in the same way. Livers and kidneys were kept frozen until analysed.

Spectrophotometric studies. Spectra of the vitamin D derivatives were measured in a Unicam SP. 700 spectrophotometer with ethanol as solvent.

Preparation of sample. The details given will be those suitable for the assay of liver and the minor changes appropriate for other tissues will be noted in the text.

Saponification. Since vitamins D_2 and D_3 could be measured separately, vitamin D_3 served as an internal standard if vitamin D_2 was to be determined and vice versa. Thus the vitamin D standard was added before saponification in an amount similar to that expected in the sample. The liver was chopped and saponified for 30min. with 50ml. of ethanol and 5ml. of saturated KOH. Water (50ml.) was added and the mixture extracted with three portions of diethyl ether (100ml. and 2×50ml.). The

ether was washed free of alkali, passed through anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure.

Precipitation of sterols. The unsaponifiable matter was dissolved in 5ml. of absolute methanol and sterols were precipitated by the addition of 5ml. of 3% (w/v) digitonin solution in 95% methanol and of 0·85ml. of water. After standing overnight, the mixture was cooled to −4° and extracted three times with 5ml. of light petroleum.

Conversion of vitamin A into anhydrovitamin A. The light petroleum was evaporated off in a stream of N_2 and the residue dissolved in 10ml. of ethanolic 33mN-HCl and left in the dark for 30min. according to the method of Barua & Rao (1964). After 30min. 10ml. of water and sufficient $NaHCO_3$ to neutralize the HCl were added and the solution was extracted three times with 20ml. of diethyl ether. The ether was washed three times with water and evaporated to dryness under N_2 or reduced pressure. This treatment was not necessary when the total amount of vitamin A was low, as, for example, in kidney and serum.

First thin-layer chromatography. The residue from the ether extracts was dissolved in a small volume of light petroleum and applied in a strip to a plate coated with Kieselgel G (E. Merck A.-G., Darmstadt, Germany) with a marker of vitamin D at one side of the plate. The chromatogram was developed with chloroform, after which the edge bearing the marker was sprayed with $SbCl_3$ in chloroform. The adsorbent opposite the marker was scraped off the plate into a small sintered-glass funnel and eluted with ethanol and diethyl ether. The eluate was evaporated to dryness under a stream of N_2. At this stage a generous width of adsorbent was removed, a little above and below that indicated by the marker spot. Vitamins D_2 and D_3 did not clearly separate in this system but stratified within the spot with vitamin D_2 forming the leading edge.

Treatment with antimony trichloride. The residue from the eluate was dissolved in 0·2ml. of chloroform and 2ml. of a 20% (w/v) $SbCl_3$ solution in chloroform was added with shaking. Small amounts of vitamin A were usually present and gave a blue colour but did not interfere with the assay. After 1min. at room temperature the reaction was stopped by the addition of 3ml. of 40% (w/v) tartaric acid in water and the suspension was shaken until two clear phases were obtained. Prolongation of the reaction time beyond 1min. resulted in deterioration of the desired product. The mixture was extracted twice with 5ml. of light petroleum and the combined extract washed three times with an equal volume of water and finally evaporated to a small volume under a stream of N_2.

Second thin-layer chromatography. The combined extract was applied to a thin-layer plate and developed as before except that the marker was vitamin D_2 or D_3 which had been treated with $SbCl_3$ as described above. The position of the vitamin was found by spraying the whole plate with a 0·001% solution of rhodamine in water and examining under u.v. light (Hanovia, model 16). The vitamin appeared as a dark area and this was carefully marked, removed from the plate and eluted as before. This method of detecting vitamin D was not useful for the unaltered vitamin but after treatment with $SbCl_3$ as little as 0·2μg. could be detected in this way.

Gas–liquid chromatography. The residue from the eluate was dissolved in a volume of light petroleum so that not more than 2·5μg. or less than 0·2μg. could be conveniently applied to a small stainless-steel gauze pad with a Microcap disposable micropipette (Shandon Scientific Co. Ltd., London, S.W.7). After 30sec. at room temperature the pad was applied to a Pye Argon Chromatograph under the following conditions: silicon-treated glass column (4ft. long; 4mm. internal diam.), packed with silicone-treated Celite with a 3% coating of silicone oil; temperature 230°; voltage 1750v; gas flow 100ml./min.

Vitamins D_2 and D_3 emerged as separate peaks and, from peak areas, the unknown was estimated by simple proportion with reference to the internal standard.

RESULTS

Ultraviolet spectra. The absorption spectra of vitamin D_2 (10μg./ml. of ethanol) before and after treatment with antimony trichloride are shown in Fig. 1. The extinction is more than doubled after treatment with antimony trichloride. It has been reported (Thibaudet, 1945) that vitamin D is converted, by treatment with antimony trichloride, into tachysterol, but the extinction curve more closely resembles that of isovitamin D as reported by Inhoffen, Brückner, Gründel & Quinkert (1954) (see Table 1). In the absence of further information the reaction product will be referred to hereafter as isovitamin D. No differences in absorption maxima or in extinction values were noted between isovitamins D_2 and D_3 prepared by treatment with antimony trichloride.

Thin-layer chromatography. The main purpose

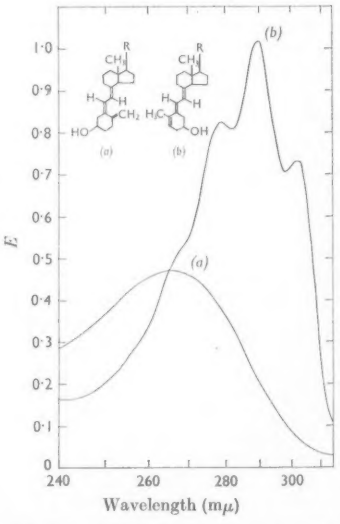

Fig. 1. Ultraviolet-absorption spectra in ethanol, and structural formulae, of vitamin D_2 (*a*) and isovitamin D_2 (*b*), the product formed by treating vitamin D with $SbCl_3$.

Table 1. *Spectral characteristics of isovitamin D_2 and the product formed by treating vitamin D_2 with antimony trichloride*

	$\lambda_{max.}$ (mμ)			Ratios of extinctions	
	A	B	C	E_B/E_A	E_B/E_C
Isovitamin D_2*	276	287	300	1·31	1·37
Vitamin D_2–$SbCl_3$	278	288	301	1·25	1·36

* Data from Inhoffen *et al.* (1954).

Table 2. *Relative retention data for vitamins D, isovitamins D and cholesterol on 3% silicone oil*

Other details of gas–liquid chromatography are given in the text.

	Relative retention volumes
Cholestane	1·0
Vitamin D_3	1·67*
	1·89†
Vitamin D_2	1·89*
	2·15†
Cholesterol	1·96
Isovitamin D_3	2·84
Isovitamin D_2	3·24

* Pyrovitamin.
† Isopyrovitamin.

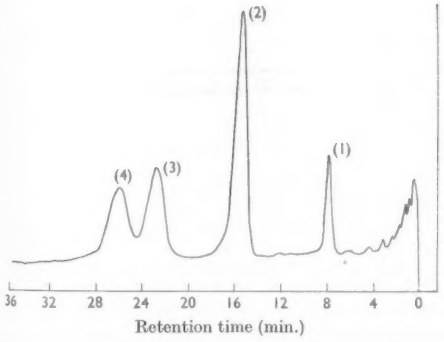

Fig. 2. Gas–liquid chromatogram of a mixture of cholestane (1), cholesterol (2), isovitamin D_3 (3) and isovitamin D_2 (4), 1μg. each. Experimental details are given in the text.

of the first thin-layer chromatography is the removal of vitamin A as anhydrovitamin A, which moves near the solvent front. Some cholesterol is usually carried over with the vitamin D and is separated on the second thin-layer chromatographing after treatment with antimony trichloride. The R_F of cholesterol (0·28) is not changed by the treatment with antimony trichloride for 1min., whereas that of vitamin D (0·36) is increased to 0·47 for isovitamin D.

Table 3. *Estimation of vitamin D_2 in the presence or absence of deficient rat liver*

Tissue	No. of estimations	Vitamin D_2 added (μg.)	Vitamin D_2 estimated (μg.)
—	14	25	26·7±0·3
Liver	10	25	25·2±0·5

Gas–liquid chromatography. Retention volumes, relative to cholestane, of vitamins D_2 and D_3, isovitamins D_2 and D_3 and cholesterol are shown in Table 2 and a typical chromatogram of cholestane, cholesterol, isovitamins D_2 and D_3, 1μg. each, is shown in Fig. 2. As little as 0·2μg. could be measured.

Application of the method. The use of the internal-standard method of calculation was tested by assaying standards alone and when added to livers of rachitic rats. In the experiments recorded in Table 3 vitamin D_2 was estimated and vitamin D_3 served as the internal standard. Good agreement was obtained between the amount estimated and that added both in the presence and absence of deficient rat liver.

Table 4 gives the results of assay of liver and kidney of rachitic rats dosed with vitamin D_2 or D_3. The disappearance of vitamin D_2 from the liver over a period of 10 days after an oral dose of 250μg. is shown by the following values (μg. of vitamin D_2/liver): day 1 after dosing, 27·4; day 2, 13·0; day 3, 5·8; day 8, 2·6; day 10, 2·5.

DISCUSSION

Treatment of vitamin D with antimony trichloride has several valuable consequences. When a gas chromatograph is not available the ultraviolet-absorption curve of isovitamin D may be useful in the determination of vitamin D. The $E^{1\%}_{1cm.}$ value is more than double that of the unaltered vitamin and the curve is such that an indication of purity may be had by calculating the extinction ratios of the maxima (Table 1). As little as 1μg./ml. may be measured in pure solutions but vitamins D_2 and D_3 cannot be estimated separately and, of course, the internal-standard method cannot be used.

The second advantage of treatment with antimony trichloride is that any vitamin A not eliminated earlier in the procedure is converted into anhydrovitamin A, which runs near the leading edge of the developing solvent (chloroform) in the

Table 4. *Assay of vitamins D_2 and D_3 in liver and kidney of dosed rats*

		Storage 24 hr. after dosing					
		Liver			Kidney		
		% of dose		Range	% of dose		Range
Dose (μg.)	No. of estimations	As D_2	As D_3		As D_2	As D_3	
Vitamin D_2 (500)	3	9·7	—	8·2–12·5	0·71	—	0·68–0·75
Vitamin D_2 (250)	6	13·1	—	8·4–20·5			
Vitamin D_3 (250)	4	—	6·4	5·1–7·2			

second thin-layer chromatographic step, well separated from isovitamin D. The differentiation of cholesterol and vitamin D is also facilitated by treatment with antimony trichloride as the R_F of cholesterol in chloroform is not affected by the treatment for 1 min. whereas that of vitamin D is appreciably increased. Consequently the sample applied to the gas chromatograph should not contain cholesterol.

Thirdly the isovitamins D_2 and D_3 produce only one peak each during gas chromatography and these are sufficiently separated from each other to permit an accurate measurement of each. Since the vitamins remain together in every step preceding gas chromatography, one may be used as an internal standard for the other. This reduces the importance of loss during sample preparation, obviates the necessity of running an additional sample with added standard and increases accuracy. Loss during assay has always been one of the great weaknesses of vitamin D determinations and has been recently discussed in detail by Chen, Terepka, Lane & Marsh (1965). The amount of internal standard and unknown should be nearly equal and this sometimes requires a preliminary run.

The assay of tissues of rats dosed with vitamins D_2 and D_3 gave results in reasonable agreement with those obtained by other methods (Kodicek & Ashby, 1960; Kodicek, 1958, 1963). Two high values, namely 15·2 and 20·5%, raised the amount of vitamin D_2 found in liver 24 hr. after dosing with 250 μg. of vitamin D_2 to a value somewhat higher than expected, but the disappearance of the vitamin over the first days after dosing is so rapid (see Results) that it is not surprising to find considerable variation at any given time.

It has been reported (Bosmann & Chen, 1964) that the livers of rats dosed with vitamin D_2 contained more antirachitic activity for chicks than could be accounted for by the vitamin D_2 content. One explanation could be the conversion by the rat of vitamin D_2 into D_3, but judging by the gas–liquid chromatography results this does not happen. When vitamin D_2 is administered vitamin D_2 is stored; similarly vitamin D_3 gives rise to vitamin D_3 (see Table 4).

The disappearance of vitamin D from the liver in the first 3 days after an oral dose was very rapid, the amount being halved on each of days 2 and 3. Thereafter the loss slowed so that in the next 7 days only 3·3 μg. disappeared, which is the lowest amount that could be estimated with any accuracy in liver. This is similar to the results of Cruickshank & Kodicek (1953), except that the vitamin D content of their animals stabilized at about 10 μg./liver and reached a plateau at 2 days after dosing.

The method described has also been successfully applied to sera of rachitic rats to which vitamin D_2 was added in a concentration of 0·5 μg./ml. of serum. However, it was found that rat faeces and chick liver contain interfering material not removed by the purification procedure described above.

We thank Mr S. G. Impey for able assistance in the biological experiments.

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Determination of Amino Acids as 2,4-Dinitrophenyl Derivatives

SERINE AND LYSINE

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(*Received 5 July* 1965)

1. Serine and lysine in complicated mixtures, such as protein hydrolysates, are dinitrophenylated quantitatively, isolated chromatographically and determined. 2. The method is accurate, moderately rapid and suitable for a number of estimations in parallel, but uses only normal laboratory apparatus.

When DNP-amino acids are partitioned between ether and aqueous acid, the majority of them are found almost entirely in the ether phase. In previous papers, methods have been described which allow the rapid separation of nearly any one of the common ether-soluble DNP-amino acids from the others on a short chromatographic column (Matheson, 1963*a*, 1965). It has already been shown that the complete series of operations (dinitrophenylation, extraction, chromatographic separation and absorptiometric estimation) can be made quantitative for valine (Matheson, 1963*b*), for aspartic acid and glutamic acid (Portugal, Green & Sutherland, 1963) and for glutamic acid and alanine (M. Bagdasarian, personal communication). It was of interest to see whether such methods could also be applied to the quantitative determination of other amino acids. For this purpose the estimation of serine and lysine was studied. These amino acids were selected because, on chromatography, their *N*-DNP derivatives have *R* values which differ from each other and from those of the ether-soluble DNP-amino acids previously estimated; further, a method for their specific determination would be of value in lipid research and in nutritional studies.

MATERIALS AND METHODS

Chemicals. Fluorodinitrobenzene was purchased from British Drug Houses Ltd., Poole, Dorset, and either used directly or after redistillation *in vacuo* (b.p. 120° at 1mm. Hg). LL-α,ϵ-Diaminopimelic acid and *meso*-α,ϵ-diaminopimelic acid were gifts from Dr Elizabeth Work, Twyford Laboratories: δ-L-hydroxylysine (containing 10–20% of δ-D-allohydroxylysine) was prepared by the method of Hamilton & Anderson (1961). DL-Serine and L-lysine hydrochloride for analytical standards were obtained from Merck, Sharp and Dohme, Rahway, N.J., U.S.A. (purity 97–103% by microbial assay), and L-serine of Sigma Grade from Sigma (London) Chemical Co. Ltd. DNP-DL-methionine sulphone and a mixture of DNP-DL-methionine (±) sulphoxides were prepared by Dr F. T. Khouw (Levy & Chung, 1955) and DNP-L-glutamine by Dr F. J. Bealing. NN^{im}-bis-(DNP)-L-histidine, *NS*-bis-(DNP)-L-cysteine and *NO*-bis-(DNP)-L-tyrosine were purchased from Sigma (London) Chemical Co. Ltd.; *NN'*-bis-(DNP)-L-cystine and *NN'*-bis-DNP-L-ornithine were bought from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A. Other chemicals were described by Matheson (1963*a*, 1965).

Phosphate buffer. This was prepared by mixing 8g. of KCl, 10·5ml. of 0·2M-NaH_2PO_4 and 87ml. of 0·2M-Na_2HPO_4. The pH was between 7·40 and 7·44.

Tris–glycine–urea–salt buffer. The solution contained 0·2M-tris, 0·2M-glycine, 6M-urea and 0·02M-NaCl and had pH about 9·5 (the exact pH is not critical).

Analysed hydrolysates. These solutions were given and analysed by Dr Gabrielle Ellinger (automatic analysis on a Beckman–Spinco analyser) by Dr R. Dawson and by Dr A. Pusztai (manual analysis; Moore, Spackman & Stein, 1958), all of the Rowett Research Institute.

Estimation of serine

Procedure. A sample containing 20–120μg. of free serine is evaporated to dryness in a test tube (2cm. diam.) with a ground-glass joint; 1ml. of 2% $NaHCO_3$ and 1·5ml. of ethanol are added and if necessary the solution is warmed slightly to dissolve any precipitated $NaHCO_3$. All subsequent operations are carried out in light from a tungsten filament bulb. Fluorodinitrobenzene (20μl.) is then added and the mixture is shaken for 0·5–1hr. at room temperature. (In the work reported in this paper, the samples were actually dinitrophenylated for 3hr. Tests showed later that, in fact, the reaction was complete for serine in less than 0·5hr.) The alcohol is removed under reduced pressure and the ether-soluble DNP-amino acids required are obtained free of FDNB,* salt etc. by extraction as described by Matheson (1963*b*), except that the combined aqueous extracts are acidified with 1ml. of 5N-HCl and that ethyl acetate is used instead of ether in the final extractions. A phosphate buffer (pH7·4)–ethyl acetate–methyl acetate (1:1:4, by vol.) mixture is prepared, and 2·5ml. of the bottom phase and 4g. of Hyflo Super-Cel are used to prepare a chromatographic column in a glass tube (1·1–1·2cm. internal diam.)

* Abbreviation: FDNB, 1-fluoro-2,4-dinitrobenzene.

as described by Matheson (1963*a*, 1965). The mixed DNP-amino acids are loaded on the column and the chromatogram is developed with the top phase of the above-mentioned mixture under gravity. The flow-rate is adjusted to 0·5–1·0 ml./min. by altering the head of top phase over the column. *N*-DNP-serine gives a compact band (*R* 0·26) which is eluted after about 44 ml. of mobile phase have emerged. It is collected in a 25 ml. standard flask containing 1 ml. of 2·5 N-triethylamine in ethyl acetate and the ultraviolet extinction is read in a 1 cm. cell at 360 mμ against air. A blank prepared from 1 ml. of triethylamine solution and 24 ml. of top phase is read similarly in the same cell, and the reading is deducted.

Comments on the analytical method. The dinitrophenylation method, used for valine, gave low yields with serine (53–65%). Extraction of the DNP-amino acids with ethyl acetate instead of ether at the final stage gave only a slightly higher yield. If DNP-serine itself were carried through the whole process there was appreciable destruction at the dinitrophenylation stage. Redistillation of the FDNB removed certain fast-moving impurities but did not improve the yield. Dinitrophenylation at 40° in an aqueous medium (Fraenkel-Conrat & Singer, 1956) increased the yield to 86–90%, though it could not be further improved. Schroeder & LeGette (1953) found that they could get high yields of DNP-amino acids if they used a much more dilute single-phase system of aqueous alcohol. The conditions they described did not produce a single phase in this Laboratory (see also Lucas, Shaw & Smith, 1963), but when modified slightly they gave an alcoholic solution of amino acid and bicarbonate in which the droplet of FDNB slowly dissolved. Such a system gave very high yields of DNP-serine from serine and reaction was complete in less than 0·5 hr.

If the sample of serine contains much acid, it will be necessary to add $NaHCO_3$ to neutralise it, as well as adding the $NaHCO_3$ required for dinitrophenylation. The pH should be about 8 (after removing any CO_2 *in vacuo*) before FDNB is added. It may be tested by adding a drop of 0·04% phenol red, which does not seem to interfere with serine determination.

The chromatographic column described by Matheson (1963*a*) will separate DNP-serine from other common DNP-amino acids but not from *N*-DNP-hydroxyproline (the *R* value, given for DNP-hydroxyproline at pH 5·4 in that paper, is incorrect). Below pH 6·7, DNP-serine and DNP-hydroxyproline will not separate. Above this pH, they do form distinct bands but their *R* values are inconveniently low. Replacement of part of the ethyl acetate by methyl acetate gave *R* values of the required size, though precipitation of a salt from the buffer sometimes occurred. The use of KCl instead of NaCl in the buffer avoided precipitation and allowed a still greater inorganic salt concentration to be employed; this further improved resolution. Table 1 gives the *R* values of a number of the DNP-amino acids closest to DNP-serine on the chromatogram. Those for diaminopimelic acid were obtained by carrying authentic LL- and *meso*-diaminopimelic acid through the analytical procedure.

Columns of this type flow very fast; it is advisable to keep the flow-rate below 1 ml./min. or resolution may be impaired. The spectrum of DNP-serine is strongly influenced by pH and, in the eluate, the absorption is of an intermediate type between the acid and alkaline spectra. Collection of the sample in triethylamine ensures that the alkaline spectrum is obtained. The absorption maximum is at 359 mμ but its value is fairly constant between 357 and 360 mμ. Determinations were all made at 360 mμ.

Table 1. *R values of DNP-amino acids on a column for isolation of DNP-serine*

The column was prepared with phosphate buffer (pH 7·40), ethyl acetate and methyl acetate as described in the text.

Compound	*R*
DNP-DL-aspartic acid	Slow
DNP-DL-glutamic acid	Slow
DNP-DL-methionine (±) sulphoxides	0·08
N-DNP-L-hydroxyproline	0·10
DNP-L-glutamine	0·15
DNP-*meso*-α,ε-diaminopimelic acid	0·20
N-DNP-L-serine	0·26
DNP-LL-α,ε-diaminopimelic acid	0·40
N-DNP-L-threonine	0·40
DNP-glycine	0·49
NN'-bis-(DNP)-L-cystine	0·57
DNP-DL-methionine sulphone	0·65
DNP-L-proline	0·68
DNP-DL-alanine	0·70

Estimation of lysine

Procedure. Dinitrophenylation is carried out as for serine though either peroxide-free diethyl ether or ethyl acetate may be used for the final extraction of the mixed DNP-amino acids. The chromatographic column is prepared from tris–glycine–urea–NaCl buffer, described above (see also Matheson, 1965), shaken with methyl acetate and ethyl acetate (1:2:3, by vol.) to form two phases. It is packed and used as described for the DNP-serine column. *NN'*-Bis-(DNP)-lysine gives a band (*R* 0·30) which has a retention volume of about 42 ml. The material is collected in a 25 ml. standard flask containing 4 ml. of ethanol and the extinction is read in a 1 cm. cell at 355 mμ and at 410 mμ against air. A blank prepared from 4 ml. of ethanol and mobile phase to 25 ml., is read similarly in the same cell and its value is deducted.

Comments on the analytical method. The dinitrophenylation method used gave good results. The reaction was complete in less than 0·5 hr. As with serine, the samples were neutralized, if necessary, before dinitrophenylation.

The chromatographic column used is similar to those described by Matheson (1965) but has an intermediate urea content (6 M). The *R* values of the DNP-amino acids closest to bis-(DNP)-lysine are given in Table 2. The *R* values for the hydroxylysines were obtained by carrying hydroxylysine, prepared from gelatin (Hamilton & Anderson, 1961), through the analytical procedure and assuming that the stronger band (81·4% of whole as measured by extinction at 355 mμ) came from the naturally occurring L-hydroxylysine and that the fainter band (18·5% of whole) came from D-allohydroxylysine. As can be seen bis-(DNP)-lysine can easily be separated from the other DNP-amino acids listed (see also Matheson, 1965) except for *NS*-bis-(DNP)-cysteine; the latter is rather close, though visibly separate. However, cysteine itself is unlikely to survive

Table 2. *R values of DNP-amino acids on a column for the isolation of* bis-*DNP-lysine*

The column was prepared with tris–glycine–urea–NaCl buffer as described in the text. *R* values of DNP derivatives of ornithine, lysine, cysteine and tyrosine were somewhat variable but the relative positions and degree of separation were fairly constant.

Compound	*R*
NN^{im}-bis-(DNP)-L-histidine	0·11
NN'-bis-(DNP)-D-allohydroxylysine	0·13
DNP-DL-phenylalanine	0·15
NN'-bis-(DNP)-L-hydroxylysine	0·18
NN'-bis-(DNP)-L-ornithine	0·18
NN'-bis-(DNP)-L-lysine	0·29
NS-bis-(DNP)-L-cysteine	0·36
NO-bis-(DNP)-L-tyrosine	0·92

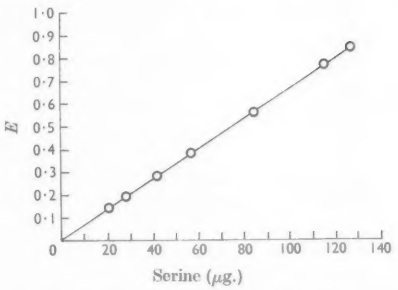

Fig. 1. Calibration curve for serine determination. The amount of serine taken (μg.) is plotted against the extinction of the final solution at 360mμ in a 1cm. cell. Details are given in the text.

hydrolysis in air to form *NS*-bis-(DNP)-cysteine on dinitrophenylation.

No triethylamine is added to the bis-(DNP)-lysine eluate before reading, since the pH of the column is high enough to ensure that the spectrum is entirely that of the alkaline form of the compound. The eluates were slightly cloudy, however, and blank extinction values tended to vary. The addition of dry ethyl acetate or methyl acetate to the eluate did not prevent this entirely, but ethanol in the receiving flask did.

The spectrum of bis-(DNP)-lysine is similar to those of many DNP-amino acids. There is a maximum at 353mμ (changing little in intensity in the range 352–355mμ) and an almost level portion around 410mμ. The wavelength 355mμ is near the end of the calibrated portion of the wavelength setting on the spectrophotometer used (model SP.600; Unicam Instruments Ltd., Cambridge). Readings were therefore taken at 410mμ also, to check whether extinction values at both wavelengths would give the same result for the lysine present and to find out if a simpler instrument, operating at a visible wavelength, could be used for determination of the yellow colour.

EXPERIMENTS AND RESULTS

Estimation of serine

Recovery experiments and standard curves. Portions of a solution, containing an accurately known amount of *N*-DNP-serine in 0·1N-hydrochloric acid, were weighed out and evaporated to dryness under vacuum. The residues were taken up in the mobile phase used for chromatography of DNP-serine, and made to 25ml. in a standard flask containing 1ml. of 2·5N-triethylamine. These solutions and a solvent blank were read at 360mμ in a 1cm. cell against air and the net extinction values were plotted against the weight of DNP-serine taken. They all fell close to a straight line. In another experiment, known amounts of DNP-serine were chromatographed and then treated similarly. Here too, the points fell close to a straight line but the slope was about 2% less. This difference may be due to a small loss during manipulation and chromatography or to traces of a coloured impurity in DNP-serine.

When known amounts of pure serine (Merck, Sharp and Dohme) were dinitrophenylated, chromatographed and determined spectrophotometrically, the results were again linear (Fig. 1). When the results were compared with those obtained on DNP-serine after chromatography, the additional loss due to dinitrophenylation and extraction was found to be about 4%. The use of the line in Fig. 1 as the standard curve for serine determination allows for this loss. From the curve, the extinction value multiplied by 147·3 gives the serine content (μg.) of the sample analysed, under the above conditions. This factor was used in the experiments recorded here, but, later, new calibration curves were set up for serine with Sigma grade L-serine (purity stated to be 100%). These gave a factor of 150·0 in one experiment and 149·6 in another instead of 147·3, but the differences are small.

Tests of the method. Hydrolysates of protein-containing material were obtained in which the amino acids had been determined by the donor by ion-exchange methods. Serine was determined in portions of the hydrolysates by the dinitrophenylation method described and the results are compared in Table 3 to the serine content calculated from the donor's result. This procedure avoided complications due to sampling errors and to variable losses during hydrolysis.

Estimation of lysine

Standard curve. The *NN'*-bis-(DNP)-lysine available (Matheson, 1963*a*) did not seem to be of

Table 3. *Comparison of serine content of hydrolysates determined by ion-exchange methods and by the dinitrophenylation procedure*

Donors: Dr Gabrielle Ellinger (E), Dr R. Dawson (D) and Dr A. Pusztai (P).

Donor	Material hydrolysed	Serine in portion analysed (μg.) Calc. from donor's value	Serine in portion analysed (μg.) By dinitrophenylation procedure	$\left(\frac{\text{Serine by dinitrophenylation}}{\text{Serine by ion-exchange}}\right) \times 100$
E	Hay	45·0	48·6	107·9
			47·3	105·1
E	Hay	46·8	49·6	106·0
			49·9	106·6
E	Maize	77·7	78·9	101·5
			79·5	102·3
E	Soya-bean protein	43·2	45·7	105·7
		64·9	69·5	107·1
		86·4	90·4	104·7
E	Fish meal	28·9	30·6	105·9
		57·7	59·5	103·1
		57·7	59·9	103·9
		115·4	117·7	102·0
D	'Conarachin' fraction of heated peanut meal 5/45	23·6	24·0	101·7
		47·1	46·7	99·1
D	'Conarachin' fraction of peanut meal	36·8	35·3	96·0
		73·5	70·1	95·4
P	Bean protein 24	25·9	26·0	100·4
		51·8	50·3	97·1
		51·8	51·7	99·8
P	Bean protein 48	43·5	43·4	99·8
			42·8	98·4

the highest purity. Its extinction value fell by 8–9% on a single passage through a column of the type described but only by 2% on a second passage. (Since the 2% loss includes losses in the manipulations needed to concentrate the material in the eluate from the first column and to free it from urea, the loss on the second column is probably very small.) A standard curve was set up on lysine itself by dinitrophenylating known amounts of pure lysine hydrochloride and submitting it to the complete procedure. The extinction values at each wavelength (355mμ and 410mμ) fall very close to straight lines (Fig. 2); the lysine content (μg.) of an unknown sample is given by 111·1 E_{355} or 315·2 E_{410}.

Tests of the method. Hydrolysates in which the lysine contents were known were obtained as before. The lysine content of portions of these hydrolysates was also determined by dinitrophenylation, either directly or else on the fast yellow material recovered from columns used for serine determination. The readings at 355mμ and at 410mμ, multiplied by the appropriate factor, gave estimates of lysine content which agreed very closely. The means of these estimates are compared with those calculated from the donor's value in Table 4.

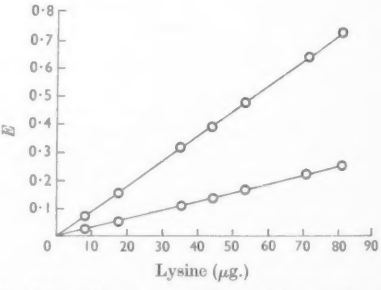

Fig. 2. Calibration curve for lysine determination. The amount of lysine taken (μg.) is plotted against the extinction of the final solution in a 1cm. cell at 355mμ (upper curve) and at 410mμ (lower curve). Details are given in the text.

DISCUSSION

Replicate determinations of serine or lysine agree very closely, the range of the results on one

Table 4. *Comparison of lysine content of hydrolysates determined by ion-exchange methods and by the dinitrophenylation procedure*

Donors were as identified in Table 3.

Donor	Material hydrolysed	Lysine in portion analysed (μg.) Calc. from donor's value	Lysine in portion analysed (μg.) By dinitrophenylation procedure	$\left(\frac{\text{Lysine by dinitrophenylation}}{\text{Lysine by ion-exchange}}\right) \times 100$
E	Hay	55·7	55·5*	99·6
E	Hay	55·9	56·0*	100·2
E	Maize	39·4	39·9*	101·3
E	Soya-bean protein	25·9	26·5	102·3
		51·8	53·7	103·7
D	'Conarachin' fraction of peanut meal GN2	30·1	30·4	101·0
		60·3	59·6	98·8
D	'Conarachin' fraction of heated peanut meal GN13	25·2	26·6	105·6
		26·9	30·1	111·9
		26·9	29·2	108·9
		50·1	54·0	107·8
		53·7	58·8	109·5
D	'Conarachin' fraction of heated peanut meal 125/5	34·2	32·5	95·0
		47·2	47·1	99·8
		67·2	67·8	100·9
D	'Conarachin' fraction of peanut meal	35·3	35·4	100·3
		70·7	71·1*	100·6
		70·7	69·5*	98·3

* Lysine determined on eluate from a column used to isolate the DNP-serine in the dinitrophenylated sample.

sample usually being about ±2%. The agreement between the serine or lysine content found by ion-exchange methods and that found by the dinitrophenylation procedure is also good, though here there are often small systematic differences between analysts. These probably relate to minor errors in calibration and are not important for the present purpose. Small random disagreements also occur occasionally but it should be remembered that the ion-exchange results given here are calculated from a single determination and that the error of this method may sometimes be in the same sense as the error for the dinitrophenylation procedure and sometimes in the opposite direction. One may conclude that the procedure described has about the same accuracy as ion-exchange methods.

Those lysine determinations marked with an asterisk were done on the DNP-amino acids which eluted rapidly from a column used to isolate DNP-serine (i.e. on the samples analysed primarily for serine). They agree with the values for lysine found by ion-exchange as closely as results on samples put directly through a column for the isolation of bis-(DNP)-lysine, so there is no appreciable loss on the DNP-serine column. The use of a single dinitrophenylation step for several amino acids may be possible in other cases also.

The lysine content determined from readings at 355mμ and 410mμ agreed well. The spectrophotometer used in this Laboratory could probably be replaced by a simple photoelectric absorptiometer with filters operating in the visible range.

It has been shown that almost any one of the common amino acids, having an ether-soluble DNP derivative, can be isolated rapidly from a mixture of all the others by partition chromatography on a short column (Matheson, 1963*a*, 1965). The columns utilize partition, mainly of ionized molecules, between organic phases formed by esters and buffered aqueous phases held stationary on kieselguhr. Braunitzer & Reuther (1956) employed a similar system in which ionic partition must also have taken place, and used it to separate DNP-amino acids. They isolated a few DNP-amino acids directly, but generally they used group separations to give mixtures which were further resolved by other solvent systems. In this Laboratory, the selective variation in R value, needed to isolate a given DNP-amino acid on a column, was achieved by varying the proportion of a particular ester in the organic phase, by adding various amounts of salts or of urea to the aqueous phase or by lowering the pH so that partition of the free acids began to play a significant part. The

dinitrophenylation procedure can probably be made quantitative for those amino acids not so far tested. If so, the method will be generally applicable. It is fairly rapid, requires only common laboratory apparatus and is suited for determining a few amino acids in a large number of samples. However, the amount of work involved depends, to a greater extent than in methods based on ion-exchange resins, on the number of different amino acids to be determined in each sample; moreover a fresh column needs to be used for each sample, since it is quicker to pack a new column (about 20min. required) than to elute the slower DNP-amino acids.

We thank Dr R. L. M. Synge for his constructive criticism of this paper, Dr Gabrielle M. Ellinger, Dr R. Dawson and Dr A. Pusztai for supplying analysed hydrolysates, Dr Elizabeth Work for her gift of LL-diaminopimelic acid and *meso*-diaminopimelic acid, Dr F. J. Bealing and Dr F. T. Khouw for certain DNP derivatives, and Miss Isobel Porter for her valuable technical assistance.

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The Incorporation of the Phosphate Esters of *N*-Substituted Aminoethanols into the Phospholipids of Brain and Liver

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(*Received* 12 *August* 1965)

1. The phosphate esters of dimethylaminoethanol and monomethylaminoethanol can be incorporated from their cytidine diphosphate esters into the phospholipids of brain and liver dispersions; the deoxycytidine nucleotides of the same bases are less effective precursors. 2. The cytidylyltransferases of brain and liver are less effective in forming the cytidine diphosphate esters of monomethylaminoethanol and dimethylaminoethanol than those of ethanolamine and choline.

The work of Kennedy and his associates (Kennedy, 1961) has unequivocally shown that phosphorylcholine and phosphorylethanolamine can be incorporated into the phospholipids of tissue preparations by a pathway involving cytidine nucleotides. It has been established that the monoesters first react with CTP to yield the CDP ester, which can then transfer the phosphorylated base to a suitable acceptor molecule, e.g. D-2,3-diglyceride, 2-acyl-1-alkenylglycerol (plasmalogenic diglyceride) or *N*-acylsphingosine (ceramide). Although deoxycytidine can replace cytidine in the nucleotide (Kennedy, Borkenhagen & Smith, 1959; Schneider & Behki, 1963) no other nucleotide base has so far proved effective (Kennedy & Weiss, 1956; Chojnacki & Korzybski, 1963). The requirement for the lipid base is much less specific (Chojnacki, 1964).

Although the enzymic steps in the cytidine pathway are the only ones capable of forming new phosphodiester bonds in phospholipids, other pathways to phosphatidyl-ethanolamine and -choline have been established in liver. Thus, although it has been known for some time that injected L-serine can give rise to ethanolamine in liver (Arnstein, 1951), Bremer, Figard & Greenberg (1960) showed that L-[3-^{14}C]serine labelled lipid ethanolamine better than the free phosphorylethanolamine or CDP-ethanolamine, suggesting that the last two compounds were not intermediates. Further, Wilson, Gibson & Udenfriend (1960) demonstrated that the labelling of lipid ethanolamine from L-[3-^{14}C]serine by liver and brain slices was not significantly diminished by adding unlabelled ethanolamine or phosphorylethanolamine. The methylation of phosphatidylethanolamine to phosphatidylcholine by *S*-adenosylmethionine *in vivo* was also indicated by the experiments of Bremer *et al.* (1960). So far the introduction of the first methyl group into phosphatidylethanolamine has not been demonstrated *in vitro* though the methylation of phosphatidylmonomethylaminoethanol and phosphatidyldimethylaminoethanol by *S*-adenosylmethionine in the presence of different liver preparations has been successfully accomplished (Artom & Lofland, 1960; Bremer & Greenberg, 1961; Artom, 1964; Rehbinder & Greenberg, 1965).

Methylation of phospholipids does not occur in brain tissue preparations (Chojnacki, Korzybski & Ansell, 1964; Marshall, Chojnacki & Ansell, 1965), which would imply an exclusive dependence on the cytidine pathway for the formation of phosphatidylcholine in this tissue. Further, evidence is available for the operation of the cytidine pathway in the formation of phospholipids containing monomethylaminoethanol and dimethylaminoethanol in mammalian tissues. After injection of 2-dimethylaminoethanol into the rat, the base could be recovered in a lipid-bound form from liver (Artom & Crowder, 1949) and as its free phosphate ester from brain and liver (Ansell & Spanner, 1962). Free dimethylaminoethanol can be incorporated into the lipids of liver homogenates and slices (Artom, 1960) and can be phosphorylated by liver preparations (Artom, 1962) and brain tissue (Ansell & Spanner, 1962). Subsequently it was shown that CDP-dimethylaminoethanol can give rise to phosphatidyldimethylaminoethanol in brain and liver homogenates (Ansell & Chojnacki, 1962*a*). The present paper deals with the incorporation of monomethylaminoethanol and dimethylaminoethanol via their CDP and dCDP esters into the phospholipids of brain and liver. Some of this work has been briefly reported (Ansell & Chojnacki, 1962*a*,*b*).

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METHODS

Materials. CMP and dCMP from Sigma Chemical Co., London, S.W. 6, or Schwarz BioResearch Inc., Orangeburg, N.Y., U.S.A., were used without further purification. Ethanolamine, 2-dimethylaminoethanol, 2-monomethylaminoethanol and choline chloride were products of British Drug Houses Ltd., Poole, Dorset. Carrier-free [^{32}P]orthophosphate was purchased from The Radiochemical Centre, Amersham, Bucks. Dicyclohexylcarbodi-imide and aluminium oxide ('prepared for chromatography') were products of British Drug Houses Ltd. and were used as purchased.

Synthesis of ^{32}P-labelled phosphorylated bases

The [^{32}P]phosphate esters of choline, monomethylaminoethanol, dimethylaminoethanol and ethanolamine were prepared by methods essentially similar to those used by Riley (1944) and Schmidt (1957) for phosphorylcholine and phosphorylethanolamine. Carrier-free [^{32}P]orthophosphate (1–5mc) was added to 0·01ml. of 88% orthophosphoric acid in a small tube (1·5cm.×4·5cm.) and the solution evaporated to a syrup with an infrared lamp. After cooling, an equimolar amount of the free base (or the chloride in the case of choline) was slowly added and the thick mixture stirred. The tube was then transferred to a pear-shaped flask containing 0·2–0·5g. of P_2O_5 and the flask connected to a vacuum line. The temperature was raised and the pressure slowly increased or decreased to prevent frothing in the reaction mixture. The cooled reaction mixture was diluted with 2–3ml. of water and heated at 100° to hydrolyse any pyrophosphate to orthophosphate. At this stage, the yield of phosphate ester was checked by ascending chromatography in propan-2-ol–water–trichloroacetic acid–18N-NH_3 (75:25:5:0·3, v/v/w/v) (Ebel, 1952). A radioautograph was made to locate the ester and the percentage conversion of [^{32}P]orthophosphate calculated from a quantitative assay of the isotope in the spot. The yields of phosphate ester obtained at 10mm. Hg pressure were: 20–30% of phosphorylcholine (155°, 6–8hr.); less than 5% of phosphoryldimethylaminoethanol (110–120°, 6–8hr.); 5–10% of phosphorylmonomethylaminoethanol (120–130°, 6–8hr.); more than 50% of phosphorylethanolamine (170°, 3hr.).

Isolation of [^{32}P]*phosphorylcholine* and [^{32}P]*phosphoryldimethylaminoethanol.* The reaction mixture diluted to 5–10ml. with water was passed through a column (1cm.×10cm.) of Amberlite IR-120 (NH_4^+ form) to remove the free base, and then through Amberlite IRA-400 (formate form) to remove inorganic phosphate. Any NH_3 could be removed with Dowex 50 (H^+ or pyridinium form) and the final effluent contained only [^{32}P]phosphoryl-base.

Isolation of [^{32}P]*phosphorylethanolamine and* [^{32}P]*phosphorylmonoethylaminoethanol.* The pH of the diluted reaction mixture was adjusted to 9–10 with N-NaOH and the mixture was diluted to about 100ml. with water. The solution was applied to a column (1·5cm.×10cm.) of Dowex 1 (formate form) resin and washed through with 50ml. of water. A linear gradient of formic acid (500ml. of 0·04M-formic acid in the reservoir and 500ml. of water in the mixing chamber) was then used to elute the phosphorylated bases. They were mostly eluted as a sharp peak when 100–150ml. of eluate had passed through the column at a rate of 40ml./hr. (cf. Weisburger & Schneider, 1961; Kennedy, 1956; Chojnacki & Korzybski, 1961). After the relevant fractions had been evaporated to dryness *in vacuo*, the radioactive esters were dissolved in water, any free resin particles were removed by centrifugation and the purity of the fraction was checked by paper chromatography in Ebel's (1952) solvent. Single ninhydrin-positive (phosphorylethanolamine only), radioactive, phosphorus-containing spots were obtained.

Synthesis of CDP esters of the substituted ethanolamines

These were prepared from CMP and the [^{32}P]phosphate esters of ethanolamine and the *N*-methylaminoethanols essentially by the method of Kennedy (1956). Equimolar amounts of CMP and the radioactive ester were dissolved in pyridine–water (7:1, v/v) and dicyclohexylcarbodi-imide (30mg./mg. of CMP) was added to the clear solution. After 2 days at 37° further dicyclohexylcarbodi-imide (15mg./mg. of CMP) was added and the process usually repeated after a further 2 days. For the derivatives of ethanolamine and monomethylaminoethanol an incubation period of 6 days was used, though half this time (and two additions of dicyclohexylcarbodi-imide) were adequate for the choline and dimethylaminoethanol derivatives. The coupling efficiency was estimated as 40% for these two compounds, about 20% for the CDP-monomethylaminoethanol and about 5% for CDP-ethanolamine.

After the appropriate reaction time the semi-solid mixture was shaken well with 20ml. of water and the mixture filtered. The cloudy filtrate was extracted three times with equal volumes of diethyl ether and the clear aqueous phase evaporated *in vacuo* to about half its original volume.

Isolation of CDP-choline and CDP-dimethylaminoethanol. The pH of the ether-free solution was adjusted to 9 with N-NaOH and diluted to 100–120ml. with water. It was then applied to a column (1·5cm.×10cm.) of Dowex 1 (X8; formate form), washed through with 50ml. of water and the components of the mixture were eluted with a linear gradient of formic acid identical with that used for phosphorylethanolamine. Fractions of volume 10ml. were collected at a rate of 40ml./hr. and assayed for extinction at 280mμ and radioactivity. The monoester of the base was eluted first followed by the CDP-choline or CDP-dimethylaminoethanol. After this came unchanged CMP. The fractions containing the required compounds were pooled, evaporated to dryness *in vacuo* at room temperature and, after removal of the fine resin particles, adjusted to pH7 with 0·1N-NaOH and diluted for use.

Isolation of CDP-monomethylaminoethanol and CDP-ethanolamine. Radioautographs of paper chromatograms of the reaction mixture in these instances showed that, although there was very little of the desired compound [R_P0·1 in Ebel's (1952) solvent] very little ^{32}P-labelled monoester remained. A number of unidentified radioactive compounds was, however, present. This is presumably one of the reasons why Kennedy (1956) and Schneider & Rotherham (1958), working with dCMP, and Sanno & Tanaka (1962) obtained such low yields of CDP-ethanolamine by this method. It was therefore necessary to make a prior separation of the desired compound by large-scale paper chromatography (Chojnacki, 1964; Ansell, Chojnacki & Metcalfe, 1965). After the reaction mixture had been

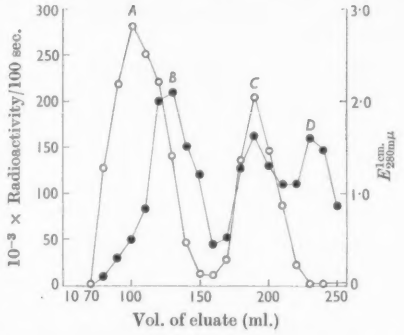

Fig. 1. Chromatography on Dowex 1 (formate form) of the reaction products formed when dicyclohexylcarbodi-imide is allowed to react with dCMP and [^{32}P]phosphorylcholine for 3 days (see the text). *A*, Unchanged [^{32}P]phosphorylcholine; *B*, unidentified material; *C*, dCDP-choline; *D*, unchanged dCMP. ○, Radioactivity; ●, $E^{1\,cm.}_{280m\mu}$.

filtered and washed with ether it was applied to Whatman no. 1 paper as a streak about 1m. in length per 100mg. of CMP originally present in the reaction mixture. After ascending chromatography in Ebel's (1952) solvent, a radioautograph of a small section of the paper was used to locate the required nucleotide. The band containing the desired compound was then cut out, washed with ether to remove the trichloroacetic acid and eluted with water. The pH of the eluate was adjusted to 9. Chromatography on a column of Dowex 1 (formate form) was then carried out in the manner described above. A non-radioactive nucleotide was also eluted from the column in appreciable amounts; this was probably dicytidine pyrophosphate or unchanged CMP.

Syntheses of deoxycytidine analogues. These were carried out exactly as described for the ribonucleotides. Separation of radioactive deoxycytidine nucleotide from unchanged phosphate monoester on Dowex 1 (formate form) was not as good as the ribose analogue but it was possible to isolate, for example, dCDP-choline from the other components of the reaction mixture (Fig. 1).

Miscellaneous methods

Solutions and spots on chromatograms containing radioactive phosphorus were assayed as described by Ansell *et al.* (1965).

Tissue preparations and incubation. Tissue was ground in an ice-cold glass pestle and mortar until a fine paste was obtained. In most experiments this was then diluted with KCl–NaF (Dawson, 1953), and 1ml. of the suspension was added to 2ml. of a mixture containing the compounds indicated in Tables 2 and 3. After a suitable period the contents of the tube were shaken with a volume of chloroform–methanol (2:1, v/v) equivalent to 20vol. of the incubated suspension. The mixture was quickly heated to the boiling point, filtered and evaporated three times to dryness *in vacuo* to break protein–lipid bonds. After dissolving in 10ml. of chloroform–methanol (2:1, v/v) the lipid solution was washed by the method of Folch, Lees & Sloane-Stanley (1957). This washed lipid was then ready for chromatography on alumina or for other investigations. In experiments in which it was desirable to investigate the radioactivity in the aqueous phase, the reaction was stopped with trichloroacetic acid (see the Results section).

For the experiments on cytidylyltransferase activity a 25% homogenate was prepared by diluting the ground tissue with water. This was incubated with CTP, [^{32}P]-phosphate esters and other components as indicated in Table 1 in a final volume of 0·4ml. After incubation the reaction was stopped by the addition of an equal volume of ice-cold 10% (w/v) trichloroacetic acid solution. A small volume of the supernatant (20–100μl.), obtained by centrifuging the precipitated homogenate, was subjected to single-dimensional chromatography in Ebel's (1952) solvent in which radioactive CDP esters were clearly separated from the monophosphate esters of the lipid bases. The CDP esters (R_F about 0·1) were located by radioautography on Kodak No-Screen X-ray film, cut out and ashed to determine the ^{32}P content.

The precipitated tissue was washed and the lipids were extracted by the method of Ansell & Dohmen (1956) to determine their ^{32}P content.

RESULTS

Formation of CDP esters of phospholipid bases. The capacity of tissues to form CDP esters of the various lipid bases was determined by incubating 0·1ml. of 25% homogenate with 0·5μmole of CTP and 0·2μmole of the [^{32}P]phosphate ester as indicated in Table 1. After precipitation with trichloroacetic acid, the radioactivity in the CDP ester formed was measured and compared with the radioactivity in the original amount of [^{32}P]phosphate ester of the lipid base added to the reaction mixture. In some experiments the amount of the radioactivity subsequently transferred to lipids was also measured.

The percentages of added phosphate esters converted into the CDP esters and into phospholipids are given in Table 1. In brain the formation of CDP esters in 15min. was slight compared with liver, but significant. The formation of CDP-monomethylaminoethanol and CDP-dimethylaminoethanol was stimulated by the presence of CTP. Small but significant amounts of radioactivity were transferred to lipids, though with both liver and brain there was a more efficient transfer of the esters of choline and ethanolamine.

Incorporation of the radioactivity of labelled nucleotides into phospholipids. Samples of CDP and dCDP esters of the ^{32}P-labelled bases were incubated with 100mg. of a brain-cortex dispersion in a medium similar to that used by Dawson (1953) at 37° in an atmosphere of oxygen + carbon dioxide (95:5). The reaction was stopped with 3ml. of

Table 1. *Transfer of radioactivity of the* [^{32}P]*phosphate esters of N-substituted aminoethanols into CDP nucleotides and phospholipids*

Each incubation consisted of 0·1 ml. of a 25% homogenate of tissue, 0·09 ml. of 0·1 M-phosphate buffer, pH 7·6, 0·01 ml. of M-$MgCl_2$, 0·1 ml. of CTP (0·5 μmole) and 0·1 ml. of a solution of the [^{32}P]phosphate ester (0·2 μmole, 6×10^5 counts/100 sec.). After incubation for the stated time at 37°, the reaction was stopped with 0·4 ml. of 10% trichloroacetic acid.

			Transfer of radioactivity from added ester (%)			
			Into brain		Into liver	
[^{32}P]Phosphate ester	Expt. no.	Incubation time (min.)	CDP nucleotides	Phospho-lipids	CDP nucleotides	Phospho-lipids
Phosphorylethanolamine	1	15	0·8*	—	9·7	—
	2	60	—	0·04	—	0·92
Phosphorylmonomethylaminoethanol	1	15	0·1*	—	1·3	—
	2	60	—	0·03	—	0·18
Phosphoryldimethylaminoethanol	1	15	0·3*	—	1·2	—
	2	60	—	0·11	—	0·08
Phosphorylcholine	1	15	1·7*	—	2·6	—
	2	60	—	0·49	—	0·89

* Mean of two experiments.

Table 2. *Incorporation of the radioactivity of* 0·05 μ*mole of labelled nucleotide into the lipids of brain-cortex dispersions* (100 *mg. of tissue* $\equiv$ 115 μ*g. of lipid phosphorus*)

The radioactivity of the labelled nucleotide varied from $1{\cdot}5 \times 10^4$ to $8{\cdot}0 \times 10^4$ counts/100 sec. In addition to the labelled nucleotide and the tissue the following constituents were present: NaCl (52·5 mM), KCl (19 mM), KH_2PO_4 (1·18 mM), $MgCl_2$ (13·6 mM), $NaHCO_3$ (25 mM), pyruvate (4·93 mM), fumarate (5·4 mM), cytochrome *c* (32 μM), AMP (1 mM) and NaF (15·5 mM). The final volume was 3 ml. and the gas phase was $O_2 + CO_2$ (95:5). The incubation time was 1 hr. The tissue was precipitated with trichloroacetic acid and the components were determined as described in the Methods section.

	Radioactivity recovered (% of that added)		
		From medium	
Labelled nucleotide	In phospholipids	Adsorbed on charcoal	Not adsorbed
CDP-choline	44·2	13·3	19·5
dCDP-choline	12·9	43·5	30·0
CDP-dimethylaminoethanol	35·0	—	—
dCDP-dimethylaminoethanol	3·5	—	—
CDP-monoethylaminoethanol	37·4	11·1	28·6
dCDP-monomethylaminoethanol	3·0	32·4	41·7
CDP-ethanolamine	36·0	—	—
dCDP-ethanolamine	0·5	—	—

10% trichloroacetic acid and the acid extract shaken with about 50 mg. of acid-washed charcoal to distinguish between ^{32}P still bound in a nucleotide and any free [^{32}P]phosphate ester of the lipid base or inorganic [^{32}P]orthophosphate. Phospholipids were extracted from the precipitate of lipid and protein by the method of Ansell & Dohmen (1956).

Table 2 shows that 35–44% of the radioactivity present in the CDP esters of all four bases was incorporated into lipids by brain cortex. The incorporation from the dCDP esters was very much less and negligible for dCDP-ethanolamine. Only about 25% of the radioactivity in the CDP ester was released as a non-nucleotide water-soluble compound; the remainder was either in a lipid form or still as a nucleotide. These experiments extend the earlier findings of Kennedy (1961) to the cytidine compounds of bases other than ethanolamine and choline and show that the deoxycytidine compounds are much less effective agents for transferring phosphorylated bases to phospholipids in the system used.

Table 3. *Incorporation of the radioactivity of labelled nucleotides* (0·05 μmole) *into the lipids of brain and liver dispersions* (100 *mg.*)

In addition to the labelled nucleotide (radioactivity varied from $1{\cdot}5\times10^4$ to $8{\cdot}0\times10^4$ counts/100sec.) and tissue the medium was identical with that in Table 2. After incubation for 1hr. the lipids were extracted with chloroform–methanol (see the Methods section).

	Radioactivity in lipid (% of that added)					
	CDP esters			dCDP esters		
Base in nucleotide	Brain cortex	White matter	Liver	Brain cortex	White matter	Liver
Choline	38·6*	23·0	26·0	11·1*	8·1	5·1
Dimethylaminoethanol	36·3*	22·3	21·0	4·2*	2·4	1·1
Monomethylaminoethanol	36·0*	20·7	20·0	3·4*	1·4	0·5
Ethanolamine	36·0	21·6	20·7	0·5	0·5	0·2

* Mean of two experiments.

Table 3 shows that, on the basis of wet tissue weight, brain cortex was nearly twice as effective as white matter or liver in incorporating the ^{32}P-labelled ester into a lipid-bound form. For all three tissues and all four lipid bases, the percentage transfer was of the same order, indicating a low specificity for the enzymes ethanolamine phosphotransferase (EC 2.7.8.1) and choline phosphotransferase (EC 2.7.8.2). For the deoxycytidine compounds, however, the amount of incorporation was dependent on the degree of methylation of the base. The reason why this should be the determining factor for the deoxy derivatives is obscure.

Fractionation of the phospholipids formed on incubation of labelled nucleotides with brain cortex. There was no reason to suppose that ^{32}P-labelled phospholipids formed from CDP-ethanolamine or CDP-choline in brain homogenates would be other than phosphatidylethanolamine (or the corresponding plasmalogen) and phosphatidylcholine respectively (McMurray, Strickland, Berry & Rossiter, 1957). It was, however, decided to identify the lipids formed from the CDP esters of monomethylaminoethanol and dimethylaminoethanol. The lipids, equivalent to about 70mg. of tissue, were dissolved in chloroform and applied to small columns of 2·5g. of alumina. They were then eluted in a stepwise fashion with chloroform–methanol or chloroform–ethanol mixtures containing increasing amounts of water (Chojnacki & Korzybski, 1964). The radioactivity and phosphorus content of the fractions were determined and the results are shown in Fig. 2.

There were two phosphorus-containing peaks corresponding to the choline- and ethanolamine-containing lipids. This was confirmed by application of the selective hydrolysis technique of Dawson (1960) to these peaks. In the experiments with labelled CDP-ethanolamine and CDP-choline the radioactivity corresponded to the peak due to the corresponding phospholipid. The radioactive lipids obtained in the experiments with CDP-monomethylaminoethanol and CDP-dimethylaminoethanol were eluted at intermediate points between choline and ethanolamine lipids as indicated in Fig. 2. Very little of the radioactivity was incorporated into phospholipids containing ethanolamine and choline. Judged from the position of elution the radioactive phospholipids were the analogues of phosphatidyl-ethanolamine and -choline. The radioactive lipid in Fig. 2(*a*) was identified as phosphatidyl-2-dimethylaminoethanol from the following criteria: (1) When the material in the radioactive peak from the alumina column was mixed with synthetic distearoylphosphatidyl-2-dimethylaminoethanol (kindly provided by Professor E. Baer) and resubjected to chromatography on alumina, the elution of the synthetic material coincided with that of the radioactive lipid. (2) Chromatography of the total lipid extract on silicic acid paper in the chloroform–methanol solvent of Lea, Rhodes & Stoll (1955) or the di-isobutyl ketone–acetic acid–water solvent of Marinetti (1962) gave only one significantly radioactive spot. Thin-layer chromatography on silica gel G (Merck) gave similar results. (3) When the radioactive peak from the alumina column was freed from glycolipids with Florisil (Ansell & Spanner, 1963) and run on silicic acid papers or thin-layer chromatograms in the presence of increasing amounts of synthetic lipid the size of the radioactive area increased (cf. Hokin & Hokin, 1958). (4) Mild alkaline hydrolysis of the radioactive lipid by the method of Dawson (1960) gave a single water-soluble phosphate ester that behaved chromatographically in phenol–aq. ammonia in the same way as the glycerylphosphoryl ester from the synthetic lipid.

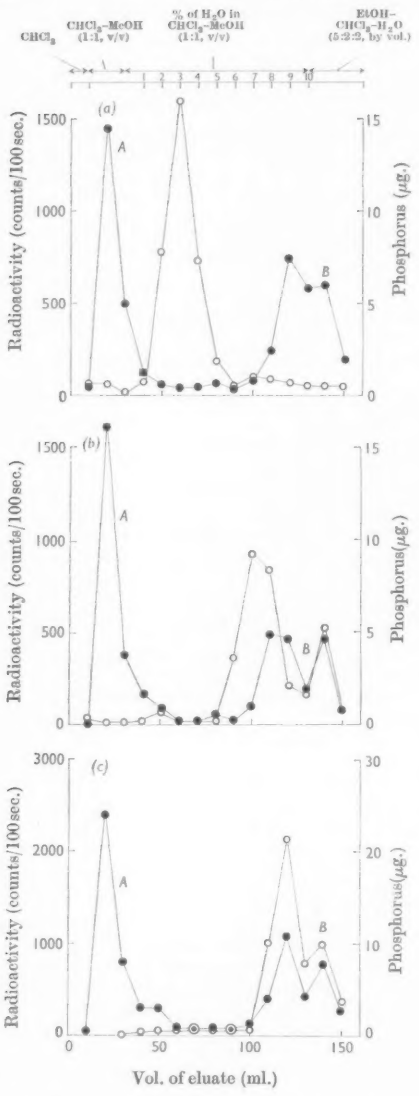

Fig. 2. Chromatography on alumina of the radioactive phospholipids formed by incubating dispersions of rat brain cortex with the CDP esters of lipid bases (see the text). An amount of lipid equivalent to about 70mg. of tissue was applied to the column in chloroform and eluted in a stepwise fashion as indicated. The radioactive lipids formed from (*a*) labelled CDP-dimethylaminoethanol, (*b*) labelled CDP-monomethylaminoethanol and (*c*) labelled CDP-ethanolamine are shown. *A*, Choline lipids; *B*, ethanolamine lipids. ○, Radioactivity; ●, phosphorus.

No synthetic phosphatidylmonomethylaminoethanol was available for comparison with the radioactive lipid synthesized from labelled CDP-monomethylaminoethanol. Its formation was assumed by analogy and the fact that it behaved as a homogeneous compound on thin-layer chromatograms and silicic acid papers.

DISCUSSION

The experiments described in this paper show that isolated adult liver and brain tissues can incorporate the labelled phosphate esters of monomethylaminoethanol and dimethylaminoethanol into the corresponding phospholipids by a pathway involving their CDP esters. The observed rate of transfer from the nucleotide to phospholipid of 0·18 μmole/g. of brain cortex tissue/hr. was the same as that found for CDP-ethanolamine and CDP-choline. It is assumed that the enzymes responsible for the transfer of phosphorylated bases are identical with or analogous to ethanolamine phosphotransferase and choline phosphotransferase, which appear to be distinct enzymes (Kennedy & Weiss, 1956). In a study with CDP-propanolamine Ansell *et al.* (1965) have shown that this ester is preferentially attacked by ethanolamine phosphotransferase, but there is a low specificity with both enzymes (Chojnacki, 1964).

From the results shown in Table 1 it is clear that ethanolamine phosphate cytidylyltransferase (EC 2.7.7.14) and choline phosphate cytidylyltransferase (EC 2.7.7.15) are more specific than the phosphotransferases. Very little activity was found towards the phosphate esters of monomethylaminoethanol and dimethylaminoethanol, particularly in brain tissue. Similarly, Ansell *et al.* (1965) have shown that cytidylyltransferase activity towards phosphorylpropanolamine was very feeble, though the transfer of the ester from CDP-propanolamine to phospholipids was as good as with CDP-ethanolamine. In short, the incorporation of the phosphate ester of a given base into phospholipids by the cytidine pathway is determined by the specificity exhibited by cytidylyltransferases.

Under the conditions used, the dCDP esters of all four lipid bases were less effective substrates for the phosphotransferases than the corresponding cytidine compounds (Table 3). The efficiency of transfer to lipid decreased with the degree of *N*-substitution so that, for example, the incorporation of phosphorylethanolamine into phospholipids of brain cortex was only 4–5% of that of phosphorylcholine when the dCDP esters were used as donors. Decreased transfer was not due to increased hydrolysis of the nucleotides in the system. Using a mixture of microsomes and mitochondria,

Kennedy *et al.* (1959) found that dCDP-choline was as effective as CDP-choline in the phosphotransferase reaction, but that, when the ethanolamine analogue was used, the CDP ester was five times as effective. Our observations on the preference for CDP-choline over dCDP-choline are in agreement with the findings of Schneider & Behki (1963), who also used rat-liver homogenates, and Chojnacki & Korzybski (1963), who used tissues from a number of species.

The fact that monomethylaminoethanol and dimethylaminoethanol can be incorporated into phospholipids via the cytidine pathway raises the issue of the importance of such a pathway in phospholipid synthesis in brain and liver. Small but significant amounts of phospholipids containing these bases are found in liver (Bremer & Greenberg, 1959), where they appear to be intermediates in the stepwise methylation to yield phosphatidylcholine (Bremer & Greenberg, 1961). On the other hand, significant amounts of the free bases or their phosphate esters have not so far been detected in animal tissues, though the free phosphate esters occur in choline-requiring mutant strains of *Neurospora crassa* (Wolf & Nyc, 1959). In brain, for example, the amount of free dimethylaminoethanol is as low as 40–80 μg./kg. (Honegger & Honegger, 1960) and no detectable amount of its phosphate ester is present (Anseli & Spanner, 1962). In fact, the formation of phosphatidyl-monomethylaminoethanol and -dimethylaminoethanol by the cytidine pathway may be of no significance in brain since their methylation to phosphatidylcholine cannot take place (Chojnacki *et al.* 1964; Marshall *et al.* 1965). It has also been shown that the formation of phosphatidylcholine by stepwise methylation is negligible *in vivo* (Ansell & Spanner, 1965). This suggests, in turn, an exclusive dependence on the cytidine pathway to phosphatidylcholine in brain, though it would not explain the origin of the choline itself. It is possible that some choline derives from serine in the brain as found by Wilson *et al.* (1960), but methylation must be part of this pathway and methylation has not been demonstrated. There is no evidence for a pathway to free choline in brain tissue and it is not impossible that all the brain choline is transferred there from the blood. The work of Groth, Bain & Pfeiffer (1958) has shown that significant amounts of intraperitoneally injected ^{14}C-labelled choline is found in the acid-soluble and lipid fractions of the brain. This suggests a direct transfer of the base from blood to brain and its subsequent phosphorylation and incorporation into phosphatidylcholine (cf. dimethylaminoethanol; Ansell & Spanner, 1962). The cytidine pathway to phosphatidylcholine, in effect, will predominate in any organ requiring exogenous choline.

Part of this work was carried out when T. C. held a British Council Scholarship. The authors also thank the Medical Research Council for financial support to T. C. during later stages of the work and for a grant towards expenses. The interest of Professor P. B. Bradley is appreciated. These investigations were carried out in the Research Laboratories attached to the Department at Uffculme Clinic, Birmingham 13, and the authors are grateful to the Management Committee for facilities provided.

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Biochem. J. (1966) **98**, 311

Uracil in Formic Acid Hydrolysates of Deoxyribonucleic Acid

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(*Received* 9 *July* 1965)

1. When DNA is hydrolysed with formic acid for 30min. at 175° and the hydrolysate is chromatographed on paper with propan-2-ol–2N-hydrochloric acid, in addition to expected ultraviolet-absorbing spots corresponding to guanine, adenine, cytosine and thymine, an ultraviolet-absorbing region with R_F similar to that of uracil can be detected. Uracil was separated from this region and identified by its spectra in acid and alkali, and by its R_F in several solvent systems. 2. Cytosine, deoxyribocytidine and deoxyribocytidylic acid similarly treated with formic acid all yielded uracil, as did a mixture of deoxyribonucleotides. 3. Approx. 4% of deoxyribonucleotide cytosine was converted into uracil by the formic acid treatment.

Formic acid (Vischer & Chargaff, 1948) and perchloric acid (Marshak & Vogel, 1951) are commonly employed for base analysis of DNA. However, in the present work the use of formic acid for determination of base ratios resulted in the appearance of an ultraviolet-absorbing region with R_F similar to that of uracil on paper chromatograms of hydrolysates of purified DNA from *Micrococcus radiodurans*. There was a complete absence of ultraviolet-absorption from the corresponding region on chromatograms of perchloric acid hydrolysates of the same DNA.

There are indications in the literature that cytosine is somewhat labile, undergoing deamination to uracil on exposure to mineral acids at elevated temperatures (Wheeler & Johnson, 1903; Vischer & Chargaff, 1948; Loring & Ploeser, 1949). This raised the possibility that uracil, derived from cytosine, was present on the chromatograms of formic acid hydrolysates of the DNA. To determine whether this phenomenon was of general occurrence hydrolysis by formic acid was extended to DNA from a variety of bacterial and animal sources, to deoxyribocytidine and deoxyribocytidylic acid and to a mixture of pure deoxyribonucleotides.

Provided that a sufficient quantity of DNA was analysed, the appearance of ultraviolet-absorbing material with an R_F similar to that of uracil on chromatograms was a constant feature of formic acid hydrolysis. The region was found to contain uracil, identified by its spectra in acid and alkali and by its R_F in a variety of solvents. It was also found that uracil is formed from cytosine and deoxyribocytidylic acid by formic acid at 175°.

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MATERIALS

DNA samples. Herring-sperm DNA [$\epsilon_{1cm.}$(P) 6700] (prepared by Dr G. Wyatt) and horse-spleen DNA [$\epsilon_{1cm.}$(P) 6600] (prepared by Dr J. D. Smith) were from this Laboratory. One sample of calf-thymus DNA (Sigma Chemical Co., St Louis, Mo., U.S.A.) was a gift from Dr M. Waring, another sample was a gift from Choay Laboratories, Paris, France, and a third sample [$\epsilon_{1cm.}$(P) 7300] was purchased from Seravac Laboratories, Colnbrook, Bucks. Purified DNA prepared by the method of Marmur (1961) from *Micrococcus lysodeikticus* was a gift from Dr M. Waring. DNA was also prepared from *Micrococcus radiodurans* by the method of Marmur (1961).

Chemicals. Guanine, cytosine, thymine and uracil were obtained from L. Light and Co. Ltd., Colnbrook, Bucks. Adenine was purchased from British Drug Houses Ltd., Poole, Dorset. Thymidine and deoxyribocytidine hydrochloride were purchased from Sigma Chemical Co. Deoxyribocytidine 5-monophosphate (disodium salt), thymidine 5-monophosphate (calcium salt), deoxyriboguanosine 5-monophosphate (disodium salt) and deoxyriboadenosine 5-monophosphate (diammonium salt, monohydrate) were gifts from Schwarz Laboratories, Orangeburg, N.Y., U.S.A. Samples of 5-hydroxymethylcytosine and 5-hydroxymethyluracil were obtained from Calbiochem, Los Angeles, Calif., U.S.A. All nucleic acid derivatives except deoxyribocytidine and the 5-hydroxymethyl compounds yielded one spot on paper chromatograms. The compounds were employed without further purification. All other chemicals were of analytical-reagent grade.

METHODS

Base ratios. The procedure employed was essentially that of Wyatt & Cohen (1953). A few milligrams of DNA

were weighed into a small Pyrex tube. Then 0·5 ml. of 88–90° (w/w) formic acid (May and Baker Ltd., Dagenham, Essex) was added and the tube was sealed off approx. 4 cm. above the surface of the liquid. The contents remained cool during sealing. The bomb tube was completely immersed in a fluidized sand bath and maintained at 175° for 30 min. The tube was opened and the formic acid was evaporated in a stream of air at 30°. The residue was dissolved in a volume of N-HCl suitable for chromatography. Occasionally the HCl solution was centrifuged. At least two portions (10 or 20 μl.) of the HCl solution were spotted on 50 cm. × 22·5 cm. sheets of Whatman no. 1 paper. Sufficient space for blanks was left adjacent to the applied spots. The chromatograms were developed in propan-2-ol–2N-HCl (Wyatt, 1951*b*) for 24–36 hr., dried in a current of warm air and photographed. The ultraviolet source was a Transparent Vitreosol mercury-vapour lamp (T/MS/534C; Thermal Syndicate Co., Wallsend, Northumberland). The filter system was described by Markham (1963). The contact printing paper was EK Duostat reflex no. 13 (Kodak Ltd., London).

Ultraviolet-absorbing spots were outlined by juxtaposition of the print and the chromatogram over a fluorescent 'white light'. The areas containing ultraviolet-absorbing material were cut out, placed in conical centrifuge tubes and eluted with 3–5 ml. of 0·1 N-HCl for 6–18 hr. The paper shreds were thoroughly mixed in the solution, and the absorption spectrum of the clear supernatant fluid obtained by centrifuging was measured in a Cary model 14 recording spectrophotometer. The estimation of base ratios was carried out according to the method of Markham (1955). The uncharacterized ultraviolet-absorbing region between cytosine and thymine with an absorption maximum at 259 mμ was eluted and calculated as uracil (ϵ_{260} 7·9 × 10^3).

DNA hydrolysis with perchloric acid. Hydrolysis, chromatography and calculation of base ratios were as described by Markham (1955).

Identification of uracil in formic acid hydrolysates of DNA. Samples (50 mg. each) of horse-spleen DNA, herring-sperm DNA and calf-thymus DNA (Seravac) and 20 mg. of *M. radiodurans* DNA were each heated with 1·5 ml. of 88–90% (w/w) formic acid in a bomb tube for 30 min. at 175°.

The tube was cooled and opened. Formic acid was evaporated in a stream of air at 30°. The residue was dissolved in 200 μl. of N-HCl and streaked on Whatman no. 3MM paper alongside a uracil marker. The papers were chromatographed in propan-2-ol–2N-HCl for 24–36 hr.

The ultraviolet-absorbing band with R_F similar to that of uracil was cut out and eluted with 2–5 ml. of water in a Perspex eluting box. The water was evaporated and the dried residue was dissolved in 100 μl. of water. Portions (50 μl.) were streaked on Whatman no. 3MM paper and chromatographed alongside a uracil marker with propan-2-ol–water plus NH_3 (Markham & Smith, 1952) for several hours. Two or more ultraviolet-absorbing bands were usually noted in this solvent. The band with R_F similar to that of uracil was cut out and eluted as described above. The eluent was evaporated and the residue was dissolved in 100 μl. of water. A portion (50 μl.) of the solution was streaked on Whatman no. 1 paper together with adjacent uracil, cytosine and thymine markers, and rechromatographed with butan-1-ol–formic acid–water (Markham & Smith, 1949). The single band with the mobility of uracil, labelled X, was cut out together with a suitable paper blank and eluted with 0·1 N-HCl. Spectra in acid and alkali were measured in a Cary recording spectrophotometer. The other 50 μl. (derived from propan-2-ol-water plus NH_3) was spotted on Whatman no. 1 paper in

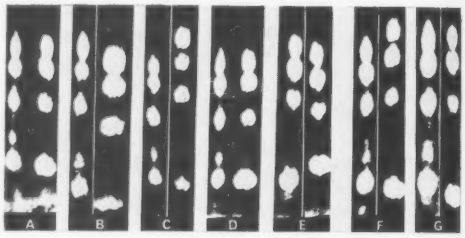

Fig. 1. Comparison of chromatograms of formic acid (left) and perchloric acid (right) hydrolysates of various deoxyribonucleic acids. Hydrolysis and chromatography procedures are described in the Methods section. The solvent was propan-2-ol–2N-HCl and the direction of flow was towards the letter designating the DNA. From top to bottom the spots represent guanine, adenine, cytosine, X (formic acid hydrolysates only) and thymine. Chromatography was not carried out for the same time; only the relative positions and the number of spots are significant. With shorter runs guanine and adenine did not separate on the chromatogram. A, herring-sperm DNA; B, *M. radiodurans* DNA; C, *M. lysodeikticus* DNA; D, horse-spleen DNA; E, calf-thymus DNA (Sigma); F, calf-thymus DNA (Choay); G, calf-thymus DNA (Seravac).

Table 1. *Purine and pyrimidine contents of formic acid hydrolysates of various deoxyribonucleic acids*

Hydrolysis and chromatography are described in the Methods section. The values listed are averages from at least duplicate determinations, each at two concentrations for chromatography. The variation between duplicate determinations was in the range 1–6%.

	Composition (moles/100 ± 1 moles of total bases)			
	Herring-sperm DNA*	Horse-spleen DNA	Calf-thymus DNA†	*M. radio-durans* DNA
Guanine	22	21	21	33
Adenine	29	29	29	17
Cytosine‡	22	20	22	33
'Uracil'§	1·2	1·8	1·4	1·5
Thymine	25	30	28	16

* Wyatt (1951*b*) gives: guanine, 22·2; adenine, 27·8; cytosine + 5-methylcytosine, 22·6; thymine, 27·5.

† Wyatt (1951*b*) gives: guanine, 21·5; adenine, 28·3; cytosine + 5-methylcytosine, 22·5; thymine, 27·8.

‡ Includes 5-methylcytosine when present.

§ Eluted ultraviolet-absorbing material with R_F (in propan-2-ol–2N-HCl) and absorption spectrum resembling those of uracil, calculated as uracil. The actual amount of uracil present may be approximately half this value.

5μl. portions and was chromatographed with a variety of marker pyrimidines in seven solvent systems.

Solvents for chromatography. These were as follows: Solvent *A*: butan-1-ol–water (43:7, v/v); 5% (v/v) of aq. NH_3 (sp.gr.0·88) was added to the solvent mixture at the bottom of the chromatography tank (Markham & Smith, 1950). Solvent *B*: butan-1-ol–water (43:7, v/v) (Markham, 1955). Solvent *C*: propan-2-ol–2N-HCl, i.e. propan-2-ol–12N-HCl–water (39:10:11, by vol.) (Wyatt, 1951*b*). Solvent *D*: propan-2-ol–acetic acid–water (6:3:1, by vol.) (Cohn, 1960). Solvent *E*: propan-2-ol–water (7:3, v/v); 0·35ml. of aq. NH_3 (sp.gr.0·88)/l. of tank volume was added to the solvent mixture at the bottom of the chromatography tank (Markham & Smith, 1952). Solvent *F*: butan-1-ol–98% (w/w) formic acid–water (77:10:13, by vol.) (Markham & Smith, 1949). Solvent *G*: butan-1-ol–acetone–water–aq. NH_3 (sp.gr.0·88) (40:50:15:3, by vol.) (Kallen, Simons & Marmur, 1962).

Identification of uracil in formic acid hydrolysates of cytosine and deoxyribocytidylic acid. Cytosine (3·34mg.) and deoxyribocytidylic acid (9·0mg.) were each treated with 0·5ml. of formic acid at 175° for 30min. All subsequent procedures were as described above. Usually from one to three ultraviolet-absorbing spots were present on the paper chromatograms. Uracil could be distinguished from cytosine, unhydrolysed deoxyribocytidine and deoxyribocytidylic acid by its characteristic R_F and different absorption spectrum.

RESULTS

All the chromatograms of the formic acid hydrolysates, but none of the perchloric acid hydrolysates, of herring-sperm DNA, *M. radiodurans* DNA, *M. lysodeikticus* DNA, horse-spleen DNA and calf-thymus DNA contained ultraviolet-absorbing

Table 2. *Spectral characteristics of X (uracil) derived from formic acid hydrolysates of various DNA samples and from formic acid-treated cytosine and deoxyribocytidylic acid*

Conditions for hydrolysis and chromatography are described in the Methods section.

	At pH 1				
Source	$\lambda_{max.}$ (mμ)	$\lambda_{min.}$ (mμ)	E_{250}/E_{260}	E_{280}/E_{260}	E at $\lambda_{max.}$/E at $\lambda_{min.}$
Uracil (literature*)	259	227	0·84	0·17	4·65
Standards					
Uracil (0·0893m-mole)	259		0·85	0·17	
Uracil (0·0223m-mole)	255–260		0·81	0·12	
Uracil (chromatographed†)	257–258	225–227	0·88	0·14	4·95
X from:					
Herring-sperm DNA	255–260		0·87	0·14	
Horse-spleen DNA	257–259	229–230	0·85	0·18	3·81
Calf-thymus DNA	259–260	227	0·87	0·16	3·80
M. radiodurans DNA	260	223	0·84		
Cytosine	259	227	0·83	0·15	5·80
Deoxyribocytidylic acid	257–259	227–229	0·89	0·18	4·20

	At pH 13					E at $\lambda_{max.}$ in alkali / E at $\lambda_{max.}$ in acid
	$\lambda_{max.}$ (mμ)	$\lambda_{min.}$ (mμ)	E_{250}/E_{260}	E_{280}/E_{260}	E at $\lambda_{max.}$/E at $\lambda_{min.}$	
Uracil (literature*)	284	241	0·71	1·41	2·86	0·75
Standards						
Uracil (0·0893m-mole)	282–284		0·71	1·33		0·67
Uracil (0·0223m-mole)	281–284	238–241	0·71	1·34	3·4	0·69
Uracil (chromatographed†)	284	241	0·71	1·31	2·5	0·74
X from:						
Herring-sperm DNA	281–283	240	0·80	1·27	2·0	0·74
Horse-spleen DNA	280–283	239–241	0·79	1·35	2·8	0·71
Calf-thymus DNA	282–284	241	0·72	1·45	2·9	0·72
Cytosine	281–283	241	0·72	1·41	2·4	0·71
Deoxyribocytidylic acid	278–282	241–242	0·69	1·27	2·2	0·73

* Calbiochem (1961).

† Treated as X and carried through the procedure described under 'Identification of uracil in formic acid hydrolysates of DNA' in the Methods section.

Table 3. *R_F values of pyrimidines and of X (uracil) from formic acid hydrolysates of various DNA samples and from formic acid-treated cytosine and deoxyribocytidylic acid*

Material	Solvent *A*	Solvent *B*	Solvent *C*	Solvent *D*	Solvent *E*	Solvent *F*	Solvent *G*
Uracil (literature)	0·19*	0·31*	0·68†			0·39*	0·34‡
Pyrimidine markers spotted for comparison							
Uracil	0·14	0·28	0·67	0·47	0·61	0·40	0·37
Cytosine	0·15	0·17	0·46	0·46	0·57	0·18	0·33
Thymine	0·32	0·41	0·77	0·67	0·69	0·54	0·55
5-Hydroxymethyluracil	0·07	0·19	0·62	0·38	0·55	0·28	0·25
5-Hydroxymethyl-cytosine	0·06	0·10	0·47	0·41	0·52	0·14	0·20
X from:							
Herring-sperm DNA	0·12	0·28	0·69	0·48	0·61	0·39	0·36
Horse-spleen DNA	0·12	0·28	0·67	0·47	0·60	0·39	0·36
Calf-thymus DNA	0·12	0·28	0·68	0·47	0·59	0·40	0·36
M. radiodurans DNA		0·29		§	0·59		§
Cytosine	0·14	0·28	0·67	0·50	0·60	0·40	0·37
Deoxyribocytidylic acid	0·14	0·28	0·67	0·49	0·61	0·40	0·37

* Markham (1955).
† Wyatt (1951*b*).
‡ Kallen *et al.* (1962).
§ Same as uracil; the solvent ran off the paper.

material, sometimes in quite discrete spots, between cytosine and thymine (Fig. 1). The material eluted from this region had an absorption maximum at 259mμ. Calculated as uracil, it averaged 1–2% of the total bases of four samples of DNA (Table 1). There was no significant difference between the ratios of the major bases obtained with perchloric acid rather than formic acid as the hydrolysing agent. The reason for this is treated in the Discussion section.

Varying the source or amount of formic acid, the type of heating bath and substitution of nitrogen for air in the bomb tube yielded essentially the same results. Prolonging the heating time increased the amount of uncharacterized ultraviolet-absorbing material.

For identification of the ultraviolet-absorbing material located between cytosine and thymine on chromatograms, larger amounts of DNA from several sources were each hydrolysed with formic acid and chromatographed on paper (see under 'Identification of uracil in formic acid hydrolysates of DNA' in the Methods section). The band with the mobility of uracil, labelled X, had the same spectra as uracil in acid and alkali (Table 2) and had essentially the same R_F as uracil in seven solvent systems (Table 3).

The same results were obtained when cytosine and deoxyribocytidylic acid were similarly treated with formic acid for 30min. at 175°, except that only one band was present in propan-2-ol–water plus ammonia. When the streak corresponding to X was eluted from the butan-1-ol–formic acid–water chromatogram it also closely resembled uracil in spectra in acid and alkali and R_F (Tables 2 and 3).

Heating cytosine, deoxyribocytidine and deoxyribocytidylic acid with formic acid for 10, 20 and 30min. produced an increasing amount of uracil (Table 4).

When a mixture of purified deoxyribonucleotides was treated with formic acid, the calculated amount of uracil formed was in the range 1·3–1·9% of the total bases (Table 5).

DISCUSSION

The absence of uracil in perchloric acid hydrolysates ruled out uracil as an intrinsic constituent of *M. radiodurans* DNA, although uracil is present in at least one species of bacteriophage DNA (Takahashi & Marmur, 1963), and also ruled out contamination by RNA or oligoribonucleotides for all of the DNA samples. There was the possibility that the ultraviolet-absorbing region between cytosine and thymine on chromatograms of formic acid hydrolysates of *M. radiodurans* contained a small amount of 5-hydroxymethylcytosine (Kallen *et al.* 1962), which, in internucleotide linkage, is destroyed by perchloric acid (Wyatt & Cohen, 1953). Another possibility was the presence of a previously undescribed base unaffected by formic acid but destroyed by perchloric acid. It was most likely, however, considering the lability of cytosine to

some acids at elevated temperatures, that a portion of the cytosine was converted into uracil.

Thus heating with 10% hydrochloric acid caused a partial conversion of cytosine into uracil (Vischer & Chargaff, 1948). Similarly Loring & Ploeser (1949) noted that cytidine refluxed with 0·4N-sulphuric acid for 48hr. was largely converted into uridine and ammonia. Vischer & Chargaff (1948) initially employed formic acid as a hydrolysing agent for RNA to minimize destruction of pyrimidine bases. In their hands the recovery of formic acid-treated cytosine was 100%; only a trace of uracil was noted on the paper chromatograms. The ratio of cytosine N recovered to total nucleotide N of formic acid-treated ribocytidylic acid never exceeded 0·8, and uracil was not observed on the paper chromatograms. When formic acid at 175° was employed to hydrolyse ox-spleen DNA and calf-thymus DNA, in no case was uracil demonstrated on the chromatograms (Chargaff, Vischer, Doniger, Green & Misaini, 1949). These investigators localized the bases on paper by reaction with Hg^{2+} followed by exposure to ammonium sulphide. Black spots of mercuric sulphide indicated the presence of the separated bases. Wyatt (1951*a*) noted the occasional presence of a trace of uracil in formic acid hydrolysates of ox-spleen DNA and herring-sperm DNA. Nothing was stated about its possible origin. Ultraviolet-absorbing spots were detected by Wyatt (1951*a*) with the aid of an ultraviolet lamp.

Examination of the propan-2-ol–2N-hydrochloric acid chromatograms of formic acid hydrolysates of DNA by ultraviolet light usually reveals at least four distinct spots (Fig. 1). Not so visible to the eye is the sometimes considerable amount of 'tailing' that occurs, increasing with the amount of hydrolysed DNA spotted and, probably, with impurities in the DNA sample. Such tailing can be revealed by photographing the chromatogram with a special ultraviolet lamp and sensitive photographic paper. The region between cytosine and

Table 4. *Conversion of cytosine into uracil by formic acid at 175°*

Cytosine, deoxyribocytidine and deoxyribocytidylic acid (0·033m-mole) were each heated with formic acid in a bomb tube at 175° for 10, 20 and 30min. Chromatography and quantitative estimation of bases were as described for DNA analysis. The results are the averages of at least duplicate determinations and are calculated from:

$$\frac{\mu\text{moles of uracil}}{\mu\text{moles of cytosine} + \mu\text{moles of uracil}} \times 100$$

The variation between duplicate analysis for uracil was in the range 1–15% and that for cytosine was in the range 1–6%.

	Percentage conversion into uracil		
Time of heating (min.)...	10	20	30
Cytosine	0·9	1·9	3·6
Deoxyribocytidine	0·8	1·6	2·5
Deoxyribocytidylic acid	0·8	2·2	4·4

Table 5. *Purine and pyrimidine base ratios of a deoxyribonucleotide mixture treated with formic acid*

Samples (10mg. each) of deoxyriboadenylic acid, deoxyriboguanylic acid, deoxyribocytidylic acid and thymidylic acid were mixed and dissolved in 10ml. of water. Portions containing 1, 2, 4, 6 and 8mg. of the deoxyribonucleotide mixture were dried and heated with formic acid in a bomb tube in the usual manner. Base ratios and uracil were measured. A portion of the unhydrolysed deoxyribonucleotide mixture was dried, and the residue was dissolved in 0·1N-HCl and chromatographed directly. The results listed are the averages of at least duplicate determinations. The variation between duplicate analysis of bases except for uracil was in the range 0–6%, with most in the range 1–3%.

	Composition (moles/100moles of total bases)						
Deoxyribonucleotide mixture spotted on chromatography paper	Untreated with formic acid*		Treated with formic acid at 175° for 30min.				
	1mg.	2mg.	0·5mg.	1·0mg.	2·0mg.	3·0mg.	4·0mg.
Guanine	20·9	21·7	20·7	21·4	20·6	20·7	21·3
Adenine	28·0	27·9	27·8	27·7	27·0	26·7	27·3
Cytosine			27·7	27·9	27·8	28·0	27·7
Deoxyribocytidylic acid	27·2	26·8					
Uracil			1·4	1·3	1·9	1·8	1·8
Thymine			22·3	22·2	22·7	22·9	22·0
Thymidylic acid	24·0	23·6					

* Adenine and guanine deoxyribonucleotides were depurinated in the 0·1N-HCl used to dissolve the mixture of deoxyribonucleotides and in propan-2-ol–2N-HCl. Only the free bases were found on chromatograms. In contrast, cytosine and thymine deoxyribonucleotides were stable in dilute acid, and free bases were absent.

thymine may appear as a narrow ultraviolet-opaque vertical streak or it may be resolved into several discrete ultraviolet-absorbing spots. With sufficient photographic developing this uncharacterized region can easily be eliminated from the print, leaving only four well-defined spots, corresponding to guanine, adenine, cytosine (plus 5-methylcytosine) and thymine. The results indicate that some of the ultraviolet-absorbing material in this region is uracil (Tables 2 and 3). Small amounts of uracil may not be observed if the amount of hydrolysate spotted is 0·25–0·5mg. of DNA or less or if the chromatograms are examined in ultraviolet light and the bases located by the naked eye. Employment of larger amounts of DNA for hydrolysis and chromatography increases the accuracy of the results and also reveals distinct and measurable amounts of uracil in the formic acid hydrolysate. Discrete spots may occasionally be observed with R_F similar to that of uracil. These, when cut out and eluted with acid, have an absorption maximum at 259mμ and an absorption spectrum resembling that of uracil.

The presence of other ultraviolet-absorbing material together with uracil on propan-2-ol–2N-hydrochloric acid chromatograms of formic acid hydrolysates of DNA renders the quantitative estimation of uracil at this stage uncertain; thus the values for uracil in Table 1 represent maxima.

To estimate the uracil specifically in formic acid hydrolysates of DNA it is necessary to elute the entire region of the paper chromatogram with R_F similar to that of uracil, and to resolve the mixture by paper chromatography in several solvent systems. Losses attendant on such a procedure would yield minimum values for uracil. When uracil was separated from other ultraviolet-absorbing constituents and estimated in this fashion about half of the material absorbing at 260mμ on propan-2-ol–2N-hydrochloric acid chromatograms in the region between cytosine and thymine was found to be uracil. Substitution of DNA by a mixture of deoxyribonucleotides allowed an estimation of uracil under conditions resembling actual DNA hydrolysis and relatively free of background ultraviolet-absorption; the uracil spots were discrete and well-outlined. Under these conditions 1·3–1·9% of the total bases appeared as uracil, a value in good agreement with the results with DNA. The uracil values for DNA are also comparable with the results of heating cytosine, deoxyribocytidine and deoxyribocytidylic acid with formic acid: approx. 3–6% of the cytosine was converted into uracil.

It is clear that uracil may be derived from cytosine or the cytosine moiety of cytosine derivatives by heating with formic acid at 175°. Fu & Bojarska (1965) obtained essentially the same results with 5-methylcytosine: approx. 5% of the methylcytosine was converted into thymine by formic acid in 30min. at 175°.

Recoveries of 96–100% of purine and pyrimidine bases after separate formic acid treatment were obtained by Wyatt (1951*b*). The same investigator, measuring individual bases from DNA, obtained reproducible results varying less than 5%. In my hands duplicate determinations of cytosine on the same or different chromatogram papers varied 1–6%. Thus the conversion of a few per cent of the cytosine into uracil may be obscured when comparisons are made of cytosine recoveries from perchloric acid and formic acid hydrolysates. The direct determination of uracil is preferable for measuring the extent of this conversion even though the variation between duplicate determinations may be as high as 15%.

These studies were supported by grants from the Office of Naval Research, the American Cancer Society (Vermont Division) and by U.S. Public Health Service Grant 103400-04 to Dr Roy Markham, Virus Research Unit, Cambridge. The work was done during the tenure of a Commonwealth Fellowship. Quantities of *Micrococcus radiodurans* were generously supplied by Dr B. Moseley and Dr H. Laser.

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The Biosynthesis of Squalene and Sterols by the Adipose Tissue of Rat, Sheep and Man

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(*Received* 2 *September* 1965)

1. The subcutaneous and omental adipose tissue of man, the epididymal fat pads of the rat and the fat tail of the Syrian sheep incorporate mevalonic acid into non-saponifiable lipids. 2. Time studies showed that the rates of decarboxylation of mevalonic acid and synthesis of non-saponifiable lipids slightly decline after 20min. but subsequently remain linear for 6hr. 3. About one-half of the incorporated radioactivity in the non-saponifiable lipids was in squalene, 20% in lanosterol and cholesterol, and the remainder in unidentified substances.

Previous studies (Vaughan, 1961) have shown that oxidative and synthetic pathways are operative in adipose tissue. However, the capacity of this tissue to synthesize sterols and other non-saponifiable lipids remained hitherto unknown. In this study, mevalonic acid, an efficient precursor of these compounds, was incubated with four types of adipose tissue, namely omental and subcutaneous human fat, the epididymal fat pads of the rat and the fat tail of the Syrian sheep. Evidence for the biosynthesis of squalene, cholesterol and lanosterol is provided.

MATERIALS AND METHODS

Tissue. Biopsy specimens of omental and abdominal subcutaneous human fat were kindly provided by Dr K. Nahra and Dr F. Suidan from the hospital of the American University of Beirut, Lebanon. The epididymal fat pads of the male rats (Wistar strain) were isolated and finely minced with a pair of scissors. Biopsy specimens were obtained from the fat tail of the Syrian sheep, *Ovis aries* var. *crassicundus*, under anaesthesia, and similarly minced.

Chemicals. DL-[^{14}C]Mevalonolactone was obtained from The Radiochemical Centre, Amersham, Bucks. Squalene (extracted from whale oil), farnesol, nerolidol and geraniol were obtained from L. Light and Co. Ltd., Colnbrook, Bucks. Cholesterol, lanosterol and related sterols were obtained from Mann Research Laboratories, New York, N.Y., U.S.A. Alumina suitable for chromatography was obtained from E. Merck A.-G., Darmstadt, Germany. Buffers were prepared as described by Durr & Cortas (1964).

Assay. The activity of the tissues was assayed first by the measurement of radioactivity in the CO_2 liberated from DL-[1-^{14}C]mevalonolactone as described by Durr & Shwayri (1964). The second assay involved the incubation of DL-[2-^{14}C]mevalonolactone with the tissue. The reaction mixture was saponified for 24hr. with 10% (w/v) KOH in methanol–water (7:3, v/v) by using 5ml./g. of tissue. The saponification mixture was concentrated to one-fifth of its volume, and the aqueous layer was exhaustively extracted with peroxide-free diethyl ether. The ether layer was washed with water until the washings were neutral and free of radioactivity. The ether layer was then dried over anhydrous Na_2SO_4; a sample was plated at infinite thinness, and the radioactivity was determined in a thin-window counter (Nuclear–Chicago Corp.) with a sensitivity of 6×10^5 counts/min./μc, and having a background counting rate of 18 counts/min. The results of duplicate incubations agreed within 2%.

Chromatography of non-saponifiable liquids. Squalene was separated from sterols and related compounds by chromatography on deactivated alumina as described by Schneider, Clayton & Bloch (1957). Cholesterol and lanosterol were separated by chromatography on deactivated alumina as described by Salokangas, Rilling & Samuels (1964).

Crystallizations. Squalene was crystallized as the hexahydrochloride as described by Heilborn, Kam & Owens (1926). I have found that farnesol, nerolidol and geraniol do not crystallize under these conditions. Cholesterol and lanosterol were crystallized from hot methanol–acetone (1:1, v/v), and their digitonides were precipitated and purified as described by Sperry & Webb (1950). Melting points were determined on a hot-stage apparatus (Fisher–Johns).

Thin-layer chromatography. Small samples of the non-saponifiable lipids were chromatographed on silica gel with ethyl acetate–hexane (1:1, v/v) as the solvent. The spots were developed by spraying the plate with conc. H_2SO_4 and heating at 100°.

Colorimetric determinations. Cholesterol was determined by the Liebermann–Burchard test (Sackett, 1925). Lanosterol was similarly determined except that the wine-red colour was read at 540mμ after 1hr.

RESULTS

Stoicheiometry and rate of mevalonolactone metabolism. Results in Table 1 show that when various

types of adipose tissue were incubated with DL-[2-^{14}C]mevalonolactone no radioactivity was detected in the respired carbon dioxide. However, a significant amount of radioactivity appeared in the non-saponifiable lipids. On the other hand, when DL-[1-^{14}C]mevalonolactone was used, the label appeared in carbon dioxide but not in the non-saponifiable lipids. These results are in agreement with the known mechanism for the metabolism of mevalonolactone (Popják & Cornforth, 1960).

Table 1. *Stoicheiometry of mevalonolactone metabolism by adipose tissue*

The incubation mixture contained 3·2 μmoles of DL-[^{14}C]mevalonolactone (1 300 000 counts/min.), 200 μmoles of glucose, 250 μmoles of phosphate buffer, pH 7·0, and 2 g. of tissue, in a final volume of 2·8 ml. Incubation was for 3 hr. at 37°.

	Radioactivity recovered in fraction (counts/min.)			
	DL-[1-^{14}C]-Mevalonolactone		DL-[2-^{14}C]-Mevalonolactone	
Adipose tissue	CO_2	Non-saponifiable lipids	CO_2	Non-saponifiable lipids
Human subcutaneous	7 680	0	0	4 800
Human omental	5 400	0	0	3 250
Rat epididymal pad	10 920	0	70	4 335
Sheep fat tail	87 600	0	210	55 250

Fig. 1 shows that the rate of carbon dioxide production paralleled that for the synthesis of non-saponifiable lipids by the fat tail of the Syrian sheep when both were simultaneously measured at 30 min. intervals. In an independent run the rates of carbon dioxide production and non-saponifiable lipid synthesis were linear for at least 6 hr. after the initial decline. Similar results were obtained with the epididymal fat pads of the rat.

Concentration of mevalonolactone. The effect of the concentration of mevalonolactone on the rate of carbon dioxide liberation from this substrate by the fat tail of the sheep and the epididymal fat pad of the rat is shown in Fig. 2. The rate of carbon dioxide production increases with increasing concentrations of mevalonolactone and slowly approaches zero-order kinetics.

Nature of the non-saponifiable lipids. Preliminary purification of the non-saponifiable lipids on deactivated alumina sharply resolved the hydrocarbon fraction from that of the sterol and other substances.

Identification of squalene. A hydrocarbon fraction of sheep non-saponifiable lipids (18 740 counts/min.)

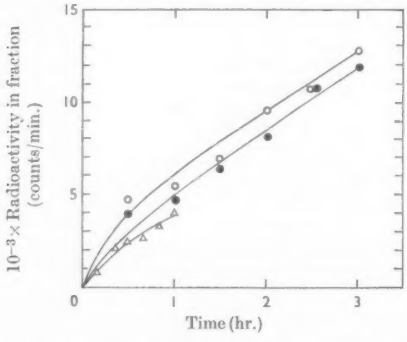

Fig. 1. Rates of CO_2 liberation and non-saponifiable lipid synthesis from mevalonolactone. The incubation mixture contained 0·8 μmole (700 000 counts/min.) of each of DL-[1-^{14}C]mevalonolactone and DL-[2-^{14}C]mevalonolactone, 1 g. of sheep fat tail, 100 μmoles of phosphate buffer, pH 7·5, and 200 μmoles of glucose, in a final volume of 3 ml. Incubation was at 37°. ●, Rate of synthesis of non-saponifiable lipids at 30 min. intervals; ○, rate of CO_2 liberation at 30 min. intervals; △, rate of CO_2 liberation at 10 min. intervals.

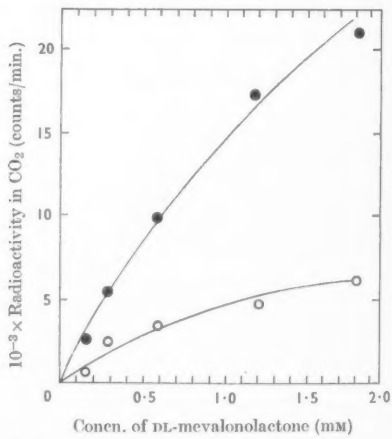

Fig. 2. Dependence of CO_2 liberation on the concentration of mevalonolactone. The incubation mixture contained 1·5 g. of tissue, DL-[1-^{14}C]mevalonolactone having a specific activity of 1 150 000 counts/min./μmole, 200 μmoles of glucose and 250 μmoles of phosphate buffer, in a final volume of 3 ml. Incubations were for 3 hr. at 37°. ●, Sheep fat tail, pH of buffer being 7·5; ○, rat epididymal fat pads, pH of buffer being 6·6.

was mixed with 0·4 ml. (334 mg.) of authentic whale squalene as carrier and was further chromatographed on deactivated alumina. The radioactivity was quantitatively recovered in the squalene peak. Squalene was subsequently crystallized as the hexahydrochloride derivative, and purified to a constant specific activity (28, 33, 33 and 33·4 counts/min./mg.). This is equivalent to a theoretical yield of 17100 counts/min. in squalene. Hence squalene represents about 92% of the hydrocarbon fraction.

The hydrocarbon fraction of the non-saponifiable lipids of the epididymal fat pad of the rat and the subcutaneous adipose tissue of man were subjected to the same treatment. Squalene accounted for 91 and 67% of the hydrocarbon fraction of the adipose tissue from rat and man respectively. Thin-layer chromatography of the hydrocarbon fraction of the various adipose tissues before adding carrier squalene showed a single orange spot (R_F 0·86). Authentic squalene gave a similar spot.

Identification and quantitative estimation of lanosterol and cholesterol. The sterol fraction (14000 counts/min.) from sheep non-saponifiable lipids was resolved by column chromatography into two fractions containing 11820 and 2740 counts/min. respectively. Fraction I (11820 counts/min.) was recovered with the peak of lanosterol. However, thin-layer chromatography of a sample gave two spots (R_F 0·73 and 0·76) having a grey and an orange colour respectively. Authentic lanosterol gave an orange spot (R_F 0·76). Crystalline lanosterol (120 mg.) was added as carrier to the lanosterol fraction and recrystallized from hot methanol–acetone (1:1, v/v) repeatedly to constant specific activity (14, 16, 18 and 17 counts/min./mg.), thereby giving a theoretical yield of 2040 counts/min. in lanosterol. This was further purified as the digitonide and recrystallized to constant specific activity (3·4, 3·8 and 3·5 counts/min./mg.), which is equivalent to a total of 1630 counts/min. Hence lanosterol accounted for about 13% of the total sterol fraction. When cholesterol was added to another sample of fraction I and crystallized, no radioactivity was detected in cholesterol. The more polar fraction (II) was recovered with the peak of cholesterol. Thin-layer chromatography of a sample of fraction II showed three violet spots (R_F 0·6, 0·63 and 0·66). Authentic cholesterol gave one violet spot (R_F 0·63). Crystalline cholesterol (120 mg.) was added to fraction II and recrystallized as described for lanosterol. It was calculated that 8% of the radioactivity in fraction II was in cholesterol. Addition of crystalline lanosterol to another sample of fraction II and recrystallization gave no radioactive lanosterol. It may be noted here that, when the non-saponifiable lipid fractions from the various tissues were treated with digitonin before the addition of carriers, the radioactivity in the digitonin-precipitable sterols was 10–20% of the total non-saponifiable lipids, which could be accounted for in lanosterol and cholesterol. Fractions I and II, when freed of cholesterol, and lanosterol still gave a faint positive Liebermann–Burchard test but no precipitate was obtained with digitonin. Attempts to identify the remaining compounds were not successful. Separate additions of dihydrocholesterol, Δ^7-dehydrocholesterol, Δ^4-cholesten-3-one, cholestan-3-one and desmosterol as carriers and subsequent recrystallizations did not account for more than 1% of the radioactivity in the non-saponifiable lipids. The sterol fractions from the adipose tissue of rat and man showed a similar pattern.

DISCUSSION

Data presented in this paper provide evidence for the incorporation of mevalonic acid into the non-saponifiable lipids of omental and subcutaneous adipose tissue of man, epididymal fat pad of rat and the fat tail of the Syrian sheep. The radioactivity in the non-saponifiable lipids derived from DL-[2-^{14}C]mevalonolactone was fractionated into squalene, cholesterol, lanosterol and unidentified substances. Whereas squalene was the major component of the non-saponifiable lipids, lanosterol and cholesterol accounted for approx. 10% of the total radioactivity in non-saponifiable lipids. The remaining fraction could not be accounted for as Δ^7-dehydrocholesterol, dihydrocholesterol, Δ^4-cholesten-3-one, cholestan-3-one or desmosterol. Ptak (1964) has extracted two highly polar types of steroids from the brown fat of the rat. These were not identified, but one of them is apparently related to corticosterone and deoxycorticosterone. The presence of several compounds accompanying lanosterol and cholesterol has been also observed in testicular tissues (Tsai, Ying & Gaylor, 1964; Salokangas *et al.* 1964). The accumulation of squalene observed in my studies is not typical of adipose tissue only. A massive accumulation of squalene was also observed when mevalonic acid was incubated with testicular tissues from rat (Tsai *et al.* 1964; Salokangas *et al.* 1964).

It was also shown that, when DL-[1-^{14}C]mevalonolactone was incubated with adipose tissue, the respired carbon dioxide was radioactive but not the non-saponifiable lipids. However, the distribution of radioactivity was reversed when DL-[2-^{14}C]-mevalonolactone was used, thereby suggesting that mevalonic acid is decarboxylated, presumably via the mechanisms operative in other mammalian tissues and bacteria (Popják & Cornforth, 1960). Durr & Shwayri (1964) have shown that for every mole of labelled carbon dioxide liberated from [1-^{14}C]mevalonolactone, 1 g.atom of labelled carbon was incorporated from [2-^{14}C]mevalonolactone into

the non-saponifiable lipids of *Lactobacillus plantarum*. This stoicheiometry in the metabolism of mevalonolactone by lactobacilli could not be demonstrated in adipose tissue. Frequently the radioactivity in the non-saponifiable lipids derived from [2-^{14}C]mevalonolactone ranged from 40 to 85% of that in the carbon dioxide liberated from [1-^{14}C]mevalonolactone. Preliminary studies indicate that the balance of radioactivity could be quantitatively accounted for in a water-soluble fraction containing at least two acids. It is possible that this fraction may contain a mixture of terpenic acids and alcohols, the biosynthesis of which occurs in rat liver (Ogilvie & Langdon, 1959; Popják, Lowe, Moore, Brown & Smith, 1959), ascites tumour (Schroepfer & Gore, 1963) and testis (Tsai *et al.* 1964). Popják, Horning, Bucher & Cornforth (1959) have shown that the metabolism of mevalonolactone can be significantly diverted to the synthesis of terpenic acids rather than squalene by withholding ascorbic acid from the incubation medium. Gosselin & Lynen (1964) have reported the synthesis of a polar lipid via pathways independent of those between farnesyl pyrophosphate and squalene.

This investigation was supported by U.S. Public Health Service Grant no. AM-05285, and by a grant from the Medical Research Committee, American University of Beirut. The author is grateful to Dr U. al-Khalidi for many stimulating discussions, and to Dr B. Dajani, Dr M. Murad and Mr K. Khoury for technical assistance.

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Synthesis and Possible Character of a High-Energy Intermediate in Bacterial Photophosphorylation

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(*Received* 23 *April* 1965)

1. In photophosphorylation with chromatophores from *Rhodospirillum rubrum*, evidence is presented for the synthesis of activated precursors of ATP in the energy-conversion system coupled to photosynthetic electron transport. 2. A significant amount of ATP is synthesized when a reaction mixture containing chromatophores and ADP is illuminated and then incubated with P_i in the dark. ATP is not synthesized to an appreciable extent, either when a reaction mixture containing chromatophores and P_i is illuminated and then incubated with ADP in the dark, or when one containing chromatophores alone is illuminated and then incubated with ADP and P_i in the dark. The amount of ATP thus synthesized is influenced markedly by concentrations of ADP. 3. The chromatophores illuminated with ADP, if allowed to stand in the dark at 30°, gradually lose the ability to form ATP with P_i in the dark. No loss of the ability occurs when the chromatophores illuminated with ADP are allowed to stand in the dark at 13° or in a frozen state. 4. Mg^{2+} is absolutely required for chromatophores to form ATP in the dark after illumination in the presence of ADP, and for the chromatophores to achieve ATP formation with P_i in the dark. 5. Antimycin A, 2-heptyl-4-hydroxyquinoline *N*-oxide and *o*-phenanthroline strongly inhibit the light-dependent acquisition of the ability to form ATP with P_i in the dark, but not the consequent ATP formation with P_i in the dark. Arsenate, 2,4-dinitrophenol, quinacrine hydrochloride, quinine hydrochloride and pyrophosphate inhibit the former or the latter, or both. Oligomycin inhibits the former somewhat more than the latter. 6. From these findings it is suggested that a high-energy intermediate is formed in photosynthetic ATP formation, and that its formation is dependent on ADP but not P_i.

Oxidative phosphorylation with mitochondria and fragments of micro-organisms is postulated to involve the formation of high-energy intermediates before the appearance of ATP. Evidence on the nature of some intermediates is scanty. Some studies indicate that phosphorylation systems contain neither ADP nor P_i (Pinchot, 1962; Webster, 1963; Webster, Smith & Hansen, 1963); others claim involvement of P_i but not ADP (Russel & Brodie, 1961; Suelter, DeLuca, Peter & Boyer, 1961; Griffiths & Chaplain, 1962*a*,*b*; Boyer, 1963; Webster *et al.* 1963; Beyer, 1964). In photophosphorylation with chloroplasts from higher plants, formation of high-energy intermediates similar to those presumably present in oxidative phosphorylation has been suggested (Kahn & Jagendorf, 1960; Avron & Sharon, 1960; Schulz & Boyer, 1961; Shen & Shen, 1962; Hind & Jagendorf, 1963; Avron, Grisaro & Sharon, 1965). In addition, Avron & Jagendorf (1959) have reported studies in which there is indirect evidence that, before reaction with P_i, ADP may participate in photosynthetic ATP formation.

With chromatophores from a facultative photoheterotroph, *Rhodospirillum rubrum*, it is known that ATP formation is coupled with the photosynthetic electron-transport system in a cyclic fashion (Horio & Kamen, 1962; Horio & Yamashita, 1963). Further, many similarities exist between partial reactions postulated for oxidative phosphorylation with mitochondria from rat liver, and those in photophosphorylation with chromatophores from *R. rubrum*, indicating that photosynthetic and oxidative phosphorylations resemble each other with respect to their mechanism of energy conversion coupled with ATP formation (Horio, Nishikawa, Katsumata & Yamashita, 1965*a*).

Evidence is here presented, from the study of chromatophores from *R. rubrum*, that there is light-dependent formation of a high-energy compound as an intermediate before the appearance of ATP, in which ADP (but not P_i) is involved in the

formation, followed by the non-photosynthetic formation of ATP from P_i and the high-energy compound.

MATERIALS AND METHODS

The cells of *R. rubrum* were grown anaerobically in the light, and chromatophores were prepared from them by grinding or ultrasonic treatment, as described previously (Horio & Kamen, 1962; Horio *et al.* 1965*a*).

Standard components of the reaction mixture were: 0·50ml. of 0·2M-glycylglycine solution adjusted to pH 8·0 with NaOH and containing 10% of sucrose (0·2M-glycylglycine–sucrose buffer, pH 8·0); 0·10ml. of 0·1M-$MgCl_2$; 0·10ml. of chromatophore suspension ($E_{880m\mu}$/ml. approx. 50); 0·10ml. of 0·075M-ascorbate solution, pH 8, and water to make 1·30ml. The reaction mixture was supplemented by 0·10ml. of 0·01M-ADP, pH 8, and 0·10ml. of 0·01-M-$^{32}P_i$, pH 8, the total volume being 1·50ml.

Reactions were carried out in test tubes of 1cm. diam. at 30°. A bank of tungsten lamps supplied illumination of approx. 1000 ft.-candles; dark conditions were obtained by inserting the test tubes in opaque cover tubes.

Standard reactions were carried out as follows. The standard components alone, plus ADP or plus $^{32}P_i$, were incubated for 5min. in the light for trials and in the dark for controls. On 'light-off', the reaction mixtures for trials and controls were completed by supplement of the missing component(s), and then reactions proceeded for 5min. in the dark with both trials and controls. Approx. 5sec. elapsed for the shift of reactions for trials from 'in the light' to 'in the dark' and those for controls from 'in the dark' to 'successively in the dark'. For brevity, the trial and control are indicated by the symbols L and D respectively. In most cases, the results obtained in this type of experiment are presented as differences between values for corresponding pairs of trials and controls, 'L minus D'. Reactions were started by adding chromatophores and ended by adding 0·50ml. of 30% (w/v) trichloroacetic acid, previously cooled in ice–water. The mixtures were allowed to stand in ice–water for at least 10min., and this was followed by centrifugation. Portions of the resulting supernatants were taken for estimation of the amounts of $^{32}P_i$ incorporated into the organic material and for identification of the resultant ^{32}P-labelled organic material (see below).

The amount of $^{32}P_i$ incorporated into organic substances was determined by the method of Nielsen & Lehninger (1950) as modified by Avron (1960), in which P_i is extracted with an organic solvent mixture. The accuracy of this assay, in particular with $^{32}P_i$ of a high radioactivity, has been re-examined and proved satisfactory under the experimental conditions used (Horio, Yamashita & Nishikawa, 1963; Horio, Stedingk & Baltscheffsky, 1965*b*).

P_i was analysed according to the method of Fiske & Subbarow (1925). The amount of P_i present in chromatophore preparations was assayed as follows. Chromatophore suspensions ($E_{880m\mu}$/ml. 50–100) were incubated with 10% (w/v) trichloroacetic acid at 0° for 10min., or boiled in N-H_2SO_4 for 7min., and then centrifuged. The resultant supernatants were used for the P_i analysis.

Identification of the organic substances containing $^{32}P_i$ was made as follows. Sample solutions were passed through charcoal columns, and the organic substances thus adsorbed were eluted with 60% (v/v) ethanol containing 3% (w/v) of NH_3. The resulting eluates were chromatographed on papers by using the solvent system reported previously (Horio, Nishikawa & Yamashita, 1964). Location of the nucleotides on the papers was made visual by illumination with an ultraviolet lamp. Identification was based on R_F values referred to those of standard nucleotides, chromatographed simultaneously. Distributions of radioactivity on the papers were measured by an Aloka, model PCS-4, paper-chromatogram scanner.

ADP, GDP, IDP and ATP were commercial preparations of the Sigma Chemical Co., St Louis, Mo., U.S.A. The ADP solutions used were shown to be sufficiently free of contaminating P_i. $^{32}P_i$ was purchased from The Radiochemical Centre, Amersham, Bucks., and purified according to the method of Suelter *et al.* (1961).

RESULTS

Survey for possible high-energy compounds photosynthesized before appearance of ATP in photophosphorylation. With chromatophores prepared from *R. rubrum* by grinding, it was demonstrated that a significant amount of $^{32}P_i$ was incorporated into the organic substance fraction, when reaction mixtures containing the standard components plus ADP were illuminated without addition of P_i and then incubated with $^{32}P_i$ in the dark (Table 1). For brevity, the first 5min. period in the light and the 5min. period immediately thereafter in the dark are expressed by the terms 'light period' and 'light-off period' respectively. When $^{32}P_i$ was present in the light period and ADP was then supplemented at the beginning of the light-off period, no significant incorporation occurred.

Horio *et al.* (1965*b*) have found that chromatophores from *R. rubrum* contain a compound in a bound state, which can be phosphorylated in the light without addition of ADP, and that the phosphorylated compound is rapidly dephosphorylated on light-off; the phosphorylated compound, if extracted from illuminated chromatophores with cold 7·5% (w/v) trichloroacetic acid, is fractionated in the organic substance fraction, and its amount reaches approx. 10mμmoles/$E_{880m\mu}$, (the amount of P_i incorporated). The phosphorylated compound thus extracted has been identified as ATP by column chromatography with Dowex 1. Horio *et al.* (1965*b*) tentatively called the compound present in chromatophores 'bound ADP'. It was found that chromatophores lost the activity for the photophosphorylation of the bound ADP without serious loss of the activity for the overall reaction of photosynthetic ATP formation with exogenous ADP, if chromatophores were centrifugally washed with 0·1M-glycylglycine–sucrose buffer, pH 8·0, containing EDTA (15μmoles/$E_{880m\mu}$ 50). This suggests that the bound ADP may be removable by the treatment with EDTA. As seen in Table 1, essentially the same results appear with the chromatophores before and after the EDTA treatment. The chroma-

Table 1. *Survey for high-energy compounds photosynthesized with chromatophores before the appearance of ATP*

The chromatophores prepared by grinding were used both with and without treatment with EDTA. ADP and $^{32}P_i$ were added to reaction mixtures in the beginning of the light period or the light-off period as indicated. L and D for the light period express experiments which were carried out in the light for trials and in the dark for controls respectively. Each of the reaction mixtures contained chromatophores of $E_{880m\mu}$ 5 and 1·0 μmole of $^{32}P_i$ of radioactivity $2{\cdot}02\times10^6$ counts/min. for the survey of high-energy compounds (nos. 1–4) and $4{\cdot}14\times10^5$ counts/min. for the overall reaction of photophosphorylation (no. 5). Reactions for nos. 1 and 5 were ended immediately after the light period. In the survey of high-energy compounds, 1·0 mμmole of ^{32}P incorporated showed 1280 counts/min. Other experimental conditions were as described in the text.

	Addition			$^{32}P_i$ incorporated (mμmoles/$E_{880m\mu}$)			
				With untreated chromatophores		With EDTA-treated chromatophores	
	Light period		Light-off period				
No.					L − D		L − D
1	$^{32}P_i$	L	—	1·6	1·5	0·2	0·2
		D		0·1		0·0	
2	$^{32}P_i$	L	ADP	2·4	0·6	1·5	0·5
		D		1·8		1·0	
3	ADP	L	$^{32}P_i$	6·5	4·9	3·8	2·7
		D		1·6		1·1	
4	none	L	$^{32}P_i$, ADP	2·1	0·1	1·3	0·1
		D		2·0		1·2	
5	$^{32}P_i$, ADP	L	—	79·0	76·5	42·0	39·5
		D		2·5		2·5	

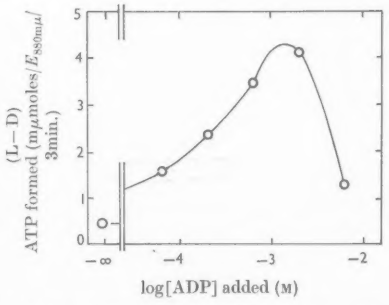

Fig. 1. Effect of concentration of ADP on photosynthesis of the ADP-dependent high-energy compound. Experimental conditions were as described in the text, except that 0·1M-$^{32}P_i$ and various concentrations of ADP were added.

tophores prepared by grinding contained approx. 1mμmole and 2mμmoles of $P_i/E_{880m\mu}$, which were extractable from the chromatophores with cold 10% (w/v) trichloroacetic acid and with boiling N-sulphuric acid respectively, and no detectable amount of P_i was present in the trichloroacetic acid extract from the EDTA-treated chromatophores. Known amounts of P_i, if added to the samples, were recovered quantitatively. The amount of the hot acid-hydrolysable phosphorus (approx. 1mμmole/$E_{880m\mu}$) accounted for one-fifth the amount that was expected from the bound ADP provided that it was ADP.

The effect of concentrations of externally-added ADP on the rate of incorporation of $^{32}P_i$ was examined with an assay system in which reactions in the light period and light-off period were carried out for 10min. and 3min. respectively. The external addition of ADP was needed for the incorporation of $^{32}P_i$, either with the non-treated or EDTA-treated chromatophores. The rate was maximum at approx. 2mM-ADP, and was markedly reduced at higher concentrations (Fig. 1).

The effect of reaction time in the light period on the incorporation of $^{32}P_i$ was studied with an assay system, in which the reaction time in the light period was varied while the time in the light-off period was fixed at 5min. (Fig. 2). The incorporation of $^{32}P_i$ attained a maximum in approx. 5min. for the light period, and then decreased with time. The effect of reaction time in the light-off period on the incorporation of $^{32}P_i$ was studied with a fixed light period of 5min. (Fig. 3). In 5–10min. for the light-off period, the incorporation of $^{32}P_i$ continued to increase without attaining a definite maximum.

By means of the paper chromatography, the substance phosphorylated in the light-off period was identified as [^{32}P]ATP; other radioactive substances were not detectable.

With chromatophores from *R. rubrum*, Geller

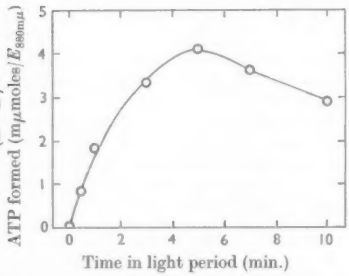

Fig. 2. Time-course of formation in the light period of the ADP-dependent high-energy compound. Experimental conditions were as described in the text, except that 0·1M-$^{32}P_i$ was added and that reaction time in the light period was varied as indicated.

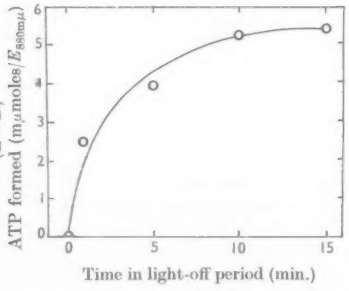

Fig. 3. Time-course of formation in the light-off period of ATP from the ADP-dependent high-energy compound and P_i. Experimental conditions were as described in the text, except that 0·1M-$^{32}P_i$ was added, and that reaction time in the light-off period was varied as indicated.

(1962, 1963) has shown that ATP is synthesized from ADP and P_i as substrates such as NADH are oxidized by molecular oxygen. The oxidative phosphorylation coupled with oxidation of NADH was completely inhibited by 10mM-cyanide, whereas the overall reaction of photophosphorylation was not influenced significantly. The incorporation of $^{32}P_i$ mentioned above was not affected by the cyanide, indicating that it was not caused by the oxidative phosphorylation coupled with the oxidation of endogenous substrates presumably present.

At 0·067mM or lower, ATP, whether added in the beginning of the light period or the light-off period, did not affect the amount of $^{32}P_i$ incorporated in both trial and control runs (Table 2).

The substance presumably synthesized in the light period in the presence of exogenous ADP will

Table 2. *Effect of ATP on photosynthesis of the ADP-dependent high-energy compound, and consequent formation in the dark of ATP*

Experimental conditions were as described in the text, except that various concentrations of ATP were supplemented in the beginning of the light period or the light-off period as indicated. L and D mean the same as in Table 1; experiments for trial (L) and control (D) were carried out in the light and dark respectively for the light period, followed by further incubation in the dark for the light-off period.

ATP added (mM)		$^{32}P_i$ incorporated (mμmoles/$E_{880m\mu}$) ATP added: Light period	$^{32}P_i$ incorporated (mμmoles/$E_{880m\mu}$) ATP added: Light-off period
0	L	6·7	
	D	2·0	
0·0067	L	6·6	6·8
	D	2·0	2·0
0·067	L	6·5	7·3
	D	3·3	3·5
0·67	L	6·9	7·7
	D	6·0	6·5

be called, henceforth, 'ADP-dependent high-energy compound'.

Stability of the ADP-dependent high-energy compound. The life time of the ADP-dependent high-energy compound was estimated as follows. The reaction mixtures containing the standard components plus ADP, which had been incubated for 5min. in the light for trials and in the dark for controls (for the light period), were allowed to stand in the dark for various lengths of time, and then $^{32}P_i$ was added and further incubated in the dark for 5min. (for the light-off period). The period intervening between the light and light-off periods is called 'intervening period'. The amount of [^{32}P]ATP thus synthesized decreased with increasing duration of the intervening period at 30°; the intervening period for approx. 40min. resulted in 50% reduction of the amount (Fig. 4). On the other hand, when the reaction mixtures were maintained at 13°, or in a frozen state during the intervening period for 45min., the amount of [^{32}P]ATP synthesized was unchanged.

To ascertain the effect of heat on the ADP-dependent high-energy compound, reaction mixtures containing the standard components plus ADP, which had been incubated for 5min. in the light period, were heated for 3min. at various temperatures in the light or in the dark for the intervening period, and then $^{32}P_i$ was added, followed by further incubation for 5min. in the dark for the light-off period (Table 3). The amount of

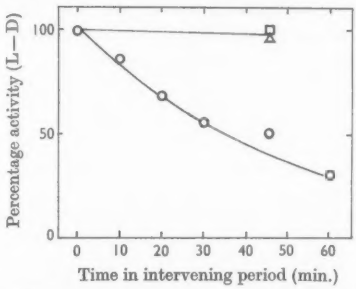

Fig. 4. Effect of temperature on the ADP-dependent high-energy compound. Experimental conditions were as described in the text, except that 0·1M-$^{32}P_i$ was added, and that reaction mixtures for the intervening period were allowed to stand in the dark at 30° (○), at 13° (△) or in a frozen state (□) for various lengths of time as indicated.

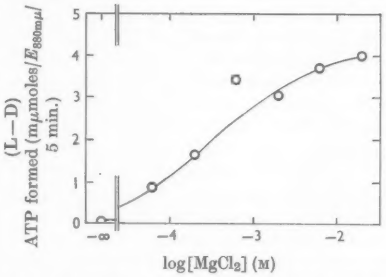

Fig. 5. Effect of Mg^{2+} on formation in the light period of the ADP-dependent high-energy compound and consequent formation in the light-off period of ATP. Various concentrations of $MgCl_2$ were added to reaction mixtures in the beginning of the light period. Other experimental conditions were as described in the text, except that 0·1M-^{32}P was added.

Table 3. *Effect of heat on the ADP-dependent high-energy compound*

Experimental conditions were as described in the text. The amount of [^{32}P]ATP synthesized in experiments having no intervening period is taken as 100%.

Temp. of system in the intervening period	Percentage activity (L—D)	
	Heated in dark	Heated in light
30°	102	123
40	88	130
50	65	112
60	16	23

[^{32}P]ATP thus synthesized tended to decrease when reaction mixtures in the intervening period were heated at 40° or higher in the dark, whereas it appeared to be unchanged when they were heated at temperatures as high as 50° in the light. Approx. 80% decrease occurred by heating at 60°, either in the light or in the dark.

Requirements of magnesium ion for photosynthesis of the ADP-dependent high-energy compound and for consequent formation in the dark of ATP. It is known that photophosphorylation with *R. rubrum* chromatophores requires Mg^{2+} (Horio *et al.* 1963). Its possible requirements for formation of the ADP-dependent high-energy compound in the light period and for the following formation of ATP from the compound and P_i in the light-off period were studied as follows. In some experiments, reaction mixtures containing chromatophores and ADP were illuminated for 5min. in the presence and absence of various concentrations of magnesium chloride, followed by supplementation with $^{32}P_i$ in the dark and by further incubation for 5min. in the dark (Fig. 5). The [^{32}P]ATP formation revealed an absolute requirement of Mg^{2+} and concentrations of the ion showed almost the same influences on both the [^{32}P]ATP formation and the overall reaction of photophosphorylation.

In other experiments, reaction mixtures containing chromatophores and ADP were illuminated for 5min. in the absence of Mg^{2+}, and then $^{32}P_i$ and Mg^{2+} were added in the dark, followed by further incubation for 5min. in the dark. [^{32}P]ATP was not synthesized to an appreciable extent. As in this series of experiments, reaction mixtures containing chromatophores and ADP were illuminated for 5min. in the presence of 6·7mM-magnesium chloride and then $^{32}P_i$ and 20mM-EDTA were added in the dark, followed by further incubation for 5min. in the dark. Again, [^{32}P]ATP was not synthesized appreciably. It seems therefore that Mg^{2+} is required for both the photosynthesis of the ADP-dependent high-energy compound and formation in the dark of [^{32}P]ATP from the high-energy compound and $^{32}P_i$.

Effects of phenazine methosulphate and ascorbate on photosynthesis of the ADP-dependent high-energy compound. Geller (1957) and Geller & Lipmann (1960) have shown that photophosphorylation with *R. rubrum* chromatophores is stimulated by phenazine methosulphate. Horio & Kamen (1962) have demonstrated that *R. rubrum* chromatophores, if depleted of endogenous reducing substances, lose the photophosphorylation ability completely, and that they regain activity if supplemented with appropriate reducing reagents such as ascorbate or PMS.* Chromatophores, if prepared by ultrasonic disruption of cells and well washed, are

* Abbreviation: PMS, phenazine methosulphate.

completely depleted of endogenous reducing substances. On the other hand, the photophosphorylation ability is stimulated to variable extents by adding ascorbate or PMS with chromatophores prepared by grinding. In the presence of an optimum concentration of PMS (0·4mM), rates of photophosphorylation (μmoles of ATP formed/$E_{880m\mu}$/hr.) are 2–5 with chromatophores prepared by grinding, and 1–2 with ones prepared by ultrasonic treatment (Horio & Yamashita, 1964; Horio *et al.* 1965*a*). By using chromatophores (prepared by ultrasonic treatment) well depleted of endogenous reducing substances, effects of ascorbate and PMS were studied on the photosynthesis of the ADP-dependent high-energy compound. Reaction mixtures containing the standard components plus ADP were incubated for 5min. in the light period in the presence and absence of various concentrations of PMS or ascorbate. Then $^{32}P_i$ was added, followed by further incubation for 5min. in the light-off period. PMS influenced the [^{32}P]ATP formation significantly (Fig. 6). In the presence of 40μM-PMS, the [^{32}P]ATP thus synthesized was approx. fivefold that in its absence, and decreased in amount at higher concentrations. The optimum concentration of PMS for this reaction was approx. one-tenth of that for the overall reaction of photophosphorylation. When omitted from the reaction mixtures in the light period and added for reactions in the light-off period, PMS had no effect on the [^{32}P]ATP synthesis. Use of ascorbate gave results similar to those with PMS but the stimulation with ascorbate was somewhat less. With the chromatophores prepared by grinding, neither PMS nor ascorbate stimulated these reactions as significantly as with the chromatophores prepared by ultrasonic treatment.

Effects of pH *on photosynthesis of the ADP-dependent high-energy compound and on consequent formation in the dark of ATP.* Reaction mixtures containing the standard components plus ADP were incubated for 2min. in the light period at various pH values, and were then re-adjusted to pH 7·9, $^{32}P_i$ was added, followed immediately by further incubation for 5min. in the light period (Fig. 7). The [^{32}P]ATP synthesis was optimum when reactions in the light period were carried out at about pH 7·2. In parallel experiments, reactions were carried out at pH 7·9 in the light period and at various pH values in the light-off period (Fig. 7). The [^{32}P]ATP synthesis was maximal when reactions in the light-off period were carried out at about pH 7. These pH optima are different from those for the overall reaction of photophosphorylation and ATP–P_i-exchange reactions in the light and dark, all of which are maximal at about pH 8 (Horio *et al.* 1965*a*).

Effects of various reagents on photosynthesis of the ADP-dependent high-energy compound and on consequent formation in the dark of ATP. Antimycin A, 2-heptyl-4-hydroxyquinoline *N*-oxide and *o*-phenanthroline, if added in the beginning of the light period, showed marked inhibitions of the [^{32}P]ATP synthesis, similar to their effects on the overall reaction of photophosphorylation (Table 4). However, these reagents, if added in the beginning

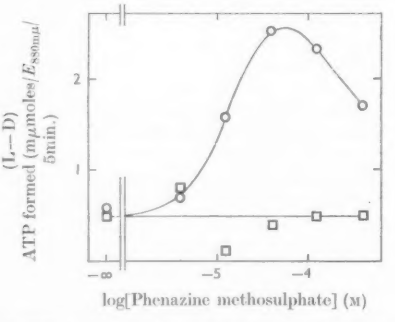

Fig. 6. Effect of phenazine methosulphate on formation in the light period of the ADP-dependent high-energy compound and consequent formation in the light-off period of ATP. Various concentrations of phenazine methosulphate were added instead of ascorbate, as indicated. Other experimental conditions were as described in the text. ○, Phenazine methosulphate added in the light period; □, phenazine methosulphate added in the light-off period.

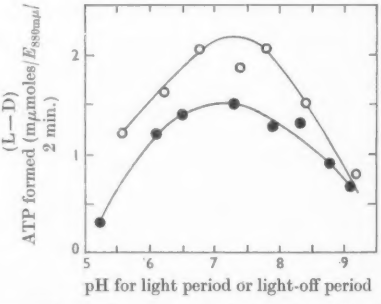

Fig. 7. Effects of pH on photosynthesis of the ADP-dependent high-energy compound and on formation in the light-off period of ATP from the high-energy compound and P_i. A buffer composed of 3,3-dimethylglutarate, tris and 2-amino-2-methylpropane-1,2-diol was used instead of glycyl-glycine–sucrose buffer. Reaction mixtures were adjusted to various pH values or pH 7·9 with 0·10ml. of 0·17M buffer for the light period, and readjusted to pH 7·9 or various pH values with 0·10ml. of 0·33M buffer for the light-off period. ○, pH varied in the light period; ●, pH varied in the light-off period.

Table 4. *Effect of various agents on photosynthesis of the ADP-dependent high-energy compound, and consequent formation in the dark of ATP*

Experimental conditions were as described in the text, except that various agents were added to reaction mixtures for the light period or the light-off period.

Addition (mM)	Percentage activity (L—D): Addition in light period	Addition in light-off period
o-Phenanthroline (0·67)	58	90
Pyrophosphate (6·7)	26	26
Adenosine (6·7)	93	101
Quinacrine–HCl (0·67)	0	1
Quinine–HCl (0·67)	40	40
Arsenate (6·7)	0	6
2,4-Dinitrophenol (3·3)	3	1
Antimycin A(0·1μg./ml.)	6	100
2-Heptyl-4-hydroxyquinoline *N*-oxide (0·67μg./ml.)	22	114
Oligomycin (0·33μg./ml.)	23	56
(1·0μg./ml.)	2	31
(3·3μg./ml.)	2	16

Table 5. *Effect of nucleotide derivatives added instead of ADP on photosynthesis of the ADP-dependent high-energy compound*

Experimental conditions were described in the text, except that various nucleotide derivatives were added instead of ADP in the beginning of the light period as indicated, and that reactions in the light-off period were carried out for 2 min.

Addition (mM)	$^{32}P_i$ incorporated (L—D) (mμmoles/$E_{880m\mu}$/2 min.)
None	0·1
ADP (0·67)	1·9
AMP (0·67)	0·1
Adenosine (0·33)	0·1
GDP (0·67)	1·8
IDP (0·67)	1·2
NAD (0·33)	0·0
NADP (0·33)	0·1
FAD (0·33)	0·0
FMN (0·33)	0·1
Riboflavine (0·33)	0·0

of the light-off period, showed no significant influence.

Quinine hydrochloride, quinacrine hydrochloride, arsenate, 2,4-dinitrophenol and pyrophosphate were inhibitory, whether added in the beginning of the light period or the light-off period. The effect of adenosine was not appreciable.

Oligomycin affected the [^{32}P]ATP synthesis; at concentrations higher than 3·3μg./ml. it inhibited the synthesis almost completely, whether added in the beginning of the light period or the light-off period. At lower concentrations, it inhibited the [^{32}P]ATP synthesis to a greater extent when added in the light period than when added in the light-off period; at 1·0μg./ml. the inhibitions were 98% for the former and 69% for the latter.

Of the nucleotide derivatives examined, GDP and IDP were substituted for ADP in the photosynthesis of the high-energy compound as well as in the overall photophosphorylation (Table 5).

DISCUSSION

In earlier studies from this Laboratory (Horio *et al.* 1965*a*), it has been shown that chromatophores from *R. rubrum* catalyse reactions for adenosine triphosphatase, ATP–P_i exchange and ADP–ATP exchange, and that these activities originate at least in part because of the reversibility of the energy-conversion system coupled to photosynthetic electron transport. From speculations applied to similar findings in the field of oxidative phosphorylation, it seems probable that some high-energy compound(s) are formed as intermediate(s) of the energy-conversion system in this bacterial photophosphorylation. In the present study, it has been found that [^{32}P]ATP is synthesized when chromatophores from *R. rubrum* are illuminated in the presence of ADP and then incubated with $^{32}P_i$ in the dark, but much less synthesis occurs when they are illuminated in the presence of $^{32}P_i$ and then incubated with ADP in the dark (Table 1).

The molar concentration of P_i present originally in chromatophores is less than one-third that of the [^{32}P]ATP synthesized in the reaction mentioned above. In addition, results are not changed by the external addition, during either the light period or the light-off period, of ATP of approx. five times the amount of the [^{32}P]ATP synthesized (Table 2). Therefore it seems unlikely that ATP is photosynthesized from the ADP externally added and the P_i originally present in chromatophores, and that the ATP thus synthesized incorporates $^{32}P_i$ by the ATP–P_i-exchange reaction in the dark. Furthermore, the amount of [^{32}P]ATP synthesized does not increase beyond a certain value with increasing time for illumination of chromatophores in the presence of an excess amount of ADP during the light period. This depends on the amount of chromatophores used, although it varies from batch to batch of chromatophore preparations, i.e. 2–6 mμmoles of [^{32}P]ATP/$E_{880m\mu}$. We conclude that chromatophores possess a component, which can be synthesized into a high-energy compound when they are illuminated in the

presence, but not absence, of ADP. One explanation for the essential requirement of ADP is that ADP is necessary for the photosynthesis of a high-energy intermediate which may or may not be a derivative of ADP. For example, ADP may be requisite for stabilization of the high-energy intermediate as it may be seen in a modification of the hypothesis of chemiosmotic energy coupling by Mitchell (1961). However, this type of explanation appears less probable because of the remarkable stability of the ADP-dependent high-energy compound at 13° and its resistance to freezing and thawing.

Of the nucleotides derivatives tested, GDP and IDP substituted for ADP in the photosynthesis of the ADP-dependent high-energy compound as well as in the overall reaction of photophosphorylation. Also, both reactions are influenced similarly by PMS, antimycin A, 2,4-dinitrophenol, oligomycin etc. Therefore it is probable that the ADP-dependent high-energy compound is synthesized as a precursor of ATP, its synthesis being coupled to the photosynthetic oxidation–reduction reactions of the cyclic electron-transport system, and that ADP functions in the same manner for the photosynthesis of the high-energy compound and the overall reaction of photosynthetic ATP formation. It seems likely that a high-energy complex is photosynthesized from ADP and the component present in chromatophores; thus [^{32}P]ATP is synthesized from ^{32}P and the complex in the dark.

With chloroplasts, Avron & Jagendorf (1959) observed that arsenate, a potent inhibitor of photophosphorylation, increases the rate of photosynthetic reduction of ferricyanide only when ADP is present, and speculated that ADP is bound in a high-energy complex first, and P_i is added as the last step in ATP formation. In studies on the fate of phosphate oxygen in photophosphorylation with chloroplasts, Avron & Sharon (1960), Avron *et al.* (1965) and Schulz & Boyer (1961) found that the terminal-bridge oxygen of ATP is furnished by its ADP moiety. This implies that the process of photophosphorylation involves phosphoryl transfer and not adenosine diphosphoryl transfer, by analogy with the known transfer reactions (Boyer, 1957). However, an alternative explanation is possible; endogenous ADP is photosynthetically phosphorylated into endogenous ATP, and then phosphoryl transfer occurs from the endogenous ATP to exogenous ADP (Horio & Yamashita, 1963; Horio *et al.* 1963). Kahn & Jagendorf (1960) reported that $^{32}P_i$ is incorporated into ATP, when chloroplasts are illuminated with $^{32}P_i$ and incubated immediately thereafter with ADP in the dark. Their finding with chloroplasts is contrary to the present finding with chromatophores. Horio *et al.* (1965*b*) found, on the other hand, that chromatophores from *R. rubrum* possess a small but definite amount of 'bound ADP', mentioned above, and that without addition of ADP it is phosphorylated in the light and the compound thus formed liberates phosphate subsequently in the dark; the reactions of the phosphorylation and dephosphorylation proceed at a similar initial rate. This also implies that exogenous ATP is formed by phosphoryl transfer from the phosphorylated 'bound ADP' to exogenous ADP.

So far there is a serious difference between the descriptions on the mechanism of photophosphorylation with higher plants and micro-organisms; photosynthetic ATP formation from ADP and P_i is essentially irreversible with chloroplasts (Jagendorf, 1962), and it is at least in part reversible with chromatophores (Horio *et al.* 1965*a*). Before any conclusion on the mechanism of the energy-conversion system of *R. rubrum* chromatophores is made, further studies are required, in particular on the relationship between the photophosphorylation of 'bound ADP' and the photosynthesis of the ADP-dependent high-energy compound.

We thank Professor Martin D. Kamen, Department of Chemistry, School of Science and Engineering, University of California-San Diego, La Jolla, Calif., U.S.A., for his helpful discussions and encouragement throughout this series of investigations. These researches have been supported by U.S. Public Health Service Grant GM-09246 from the National Institutes of Health, Bethesda, Md., U.S.A.

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Biochem. J. (1966) **98**, 330

Cobalt Toxicity and Iron Metabolism in *Neurospora crassa*

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(*Received* 16 *June* 1965)

1. Increasing concentrations of cobalt in the medium result in increased production of an iron-binding compound and a corresponding fall in catalase activity of *Neurospora crassa*. 2. Cobalt rapidly depletes the medium of iron by enhancing the rate of iron uptake by the mycelium. 3. With toxic amounts of cobalt there is a fall in bound ^{59}Fe and haem ^{59}Fe as well as a decreased incorporation of [2-^{14}C]glycine into the mycelial haem fraction. The production of the iron-binding compound precedes the fall in the iron-dependent systems mentioned. 4. The ^{59}Fe bound to the iron-binding compound acts as a better iron source for haem synthesis in cell-free extracts as compared with $^{59}FeSO_4$. 5. Cobalt inhibits iron incorporation into protoporphyrin in cell-free extracts but is not itself incorporated to an appreciable extent.

Cobalt toxicity has been related to iron deficiency in *Aspergillus niger* (Adiga, Sivarama Sastry, Venkatasubramanyam & Sarma, 1961). A direct cobalt–iron antagonism is also demonstrable in *Neurospora crassa* (Sivarama Sastry, Adiga, Venkatasubramanyam & Sarma, 1962). Healy, Cheng & McElroy (1955), in studies at the enzymic level, concluded that cobalt toxicity is comparable with straight iron deficiency in *N. crassa*. A further correlation in this organism has been the isolation of an iron-binding compound (X) from the culture fluid when the organism is grown under conditions of cobalt toxicity or straight iron deficiency (Padmanaban & Sarma, 1964). The iron complex of the iron-binding compound (XFe) serves as a good source of iron for the parent organism to maintain normal growth, catalase and a few non-haem iron enzyme activities. Further, this organic iron is incorporated into the mycelium much faster than inorganic iron or a simple chelate like ferric citrate (Padmanaban & Sarma, 1965). Preliminary studies show that this iron-binding compound belongs to the siderochrome (Bickel *et al.* 1960) class of compounds.

A study of the interference of cobalt with the iron metabolism of the mould *N. crassa* has been carried out and the results are presented below.

EXPERIMENTAL

Culture and growth conditions. *N. crassa* Em 5297a (wild) was used. The composition of the medium was as described by Sivarama Sastry *et al.* (1962). The organism was grown in 50ml. Pyrex conical flasks in 10ml. of basal medium at 30° without shaking.

Effect of cobalt on ^{59}Fe *uptake.* The organism was grown with 800μg. of cobalt/10ml. of basal medium and optimum concentrations of iron (1μg./10ml. of basal medium) as [^{59}Fe]ferric citrate. After growth for the required period, the mycelia were removed, washed free of adhering radioactivity, weighed and made into a fine suspension with 5ml. of water. A portion was used to measure the total radioactivity incorporated, by using a DSS–5 scintillation detector attached to a decade scaler (Nuclear–Chicago Corp., Des Plaines, Ill., U.S.A.). The rest of the sample was precipitated with cold trichloroacetic acid (final concn. 10%, w/v). The precipitate was washed four times with cold trichloroacetic acid and the radioactivity on the precipitate was measured after digestion with acid. The trichloroacetic acid-precipitable ^{59}Fe is referred to below as bound ^{59}Fe. The results are expressed in terms of mycelial dry weight. The dry weight/fresh weight ratio was 1:5.

Effect of cobalt on ^{59}Fe *and* [2-^{14}C]*glycine incorporation into the mycelial haem fraction.* The mycelia grown in the presence of cobalt and the appropriate tracer were washed free of adhering radioactivity and the haemin was isolated from the mycelial acetone-dried powder according to the following method of Labbe & Nishida (1957). A sample (1g.) of the acetone-dried powder was suspended in 5ml. of water, and 100ml. of a solvent mixture of acetic acid saturated with $SrCl_2$ and acetone (1:3, v/v) was added. Since insufficient haemin could be isolated to permit subsequent purification, 50mg. of carrier haemin was added. The mixture was kept overnight and then briefly heated to the boiling point of the solvent and filtered. The residue was washed thrice with acetic acid–acetone mixture and haemin was isolated from the filtrate. The radioactivity of a known amount of isolated and recrystallized haemin was taken as a measure of the radioactivity incorporated into the mycelial haem fraction.

^{14}C measurements were made in a Geiger–Müller end-window counter attached to a decade scaler (Nuclear–Chicago Corp.). Appropriate corrections due to self-absorption, background and radioactive decay were applied.

Effect of cobalt on ^{59}Fe *incorporation into protoporphyrin in cell-free extracts.* The procedure employed was essentially the same as described by Nishida & Labbe (1959) involving the estimation of radioactivity associated with the enzymically formed haemin from ^{59}Fe and protoporphyrin. The 72hr.-old normal mycelia (10g. fresh wt.) were ground in 50ml. of 0·1M-phosphate buffer, pH7·5, in the presence of 100mg. of Tween 20 and glass powder by using a pestle and mortar and centrifuged at 15000*g* for 20min. after keeping the homogenate stirred for 1hr. This enzyme preparation was incubated with the other constituents in amounts as used by Labbe & Hubbard (1961) for 2hr. under N_2 at 37° in a Dubnoff metabolic shaker. The reaction was stopped with the acetic acid–acetone mixture, and haemin was isolated after adding 10ml. of carrier blood, by the procedure described above. The recrystallized haemin was dissolved in alkaline pyridine and portions were used to measure radioactivity and haemin content.

Catalase assay. Catalase activity was determined in phosphate buffer extracts of the mycelia by the procedure described by Ramachandran & Sarma (1954).

Protein content. The method of Lowry, Rosebrough, Farr & Randall (1951) was used to estimate protein in the enzyme samples.

Estimation and isolation of the iron-binding compound For estimation the method employed by Neilands (1957) was used. To 3ml. of the culture fluid, 1ml. of $FeCl_3,6H_2O$ (1mg. of iron/ml.) solution was added and centrifuged. The extinction of the supernatant was measured at 440mμ, since XFe has an absorption maximum at this wavelength. The iron-binding compound (X) was isolated as the iron complex (XFe) from the cobalt-toxic culture fluid by the addition of $FeCl_3,6H_2O$ followed by $(NH_4)_2SO_4$ saturation, benzyl alcohol extraction and subsequent purification as described by Padmanaban & Sarma (1964). X^{59}Fe was isolated after adding $^{59}FeCl_3$ directly to the cobalt-toxic culture fluid. The iron content in XFe was estimated, after wet digestion of the sample, by the *o*-phenanthroline method (Sandell, 1950).

RESULTS

A reciprocal relationship exists in *N. crassa* between the production of the iron-binding compound and catalase activity and this is governed by the iron status of the growth medium (Padmanaban & Sarma, 1965). Increasing concentrations of cobalt cause an increase in the production of the iron-binding compound and a corresponding fall in catalase activity (Table 1). The fall in the production of the iron-binding compound at the highest concentration of cobalt employed could be due to the severe inhibition of the growth of the organism.

Thus the results presented in Table 1 simulate conditions of iron deficiency and it was decided to examine whether cobalt causes an iron deficiency by inhibiting the uptake of this essential metal by the organism. Cobalt does not inhibit iron uptake but enhances instead the rate of incorporation (Table 2). The ^{59}Fe incorporated by the mycelium grown with cobalt is double the corresponding value for the normal mycelium at the end of 24hr. growth. This indicates that cobalt enhances the rate of ^{59}Fe incorporation into the mycelium in addition to its growth-inhibitory effects, and this contributes to the considerably higher iron concentration, when

Table 1. *Effect of increasing concentrations of cobalt on growth, catalase activity and iron-binding compound production in* N. crassa *at the end of 72hr. growth*

Experimental details are given in the text.

Cobalt added (μg./10ml. of medium)	Growth (mg. dry wt.)	Catalase activity (ml. of 10mM-$KMnO_4$ consumed/mg. of protein/5min.)	Iron-binding compound ($E_{440m\mu}$)
0	42·8	50·2	0·03
50	46·0	35·4	0·10
200	37·2	20·2	0·16
600	28·1	13·1	0·20
800	22·0	9·0	0·22
1000	13·2	5·7	0·07

Table 2. *Effect of cobalt on total and bound* ^{59}Fe *in the mycelia as a function of growth period in* N. crassa

The basal medium contained in 10ml. 24·6mμc (1·0μg. of iron) as [^{59}Fe]ferric citrate. Experimental details are given in the text.

Period of growth (hr.)	Normal: Total ^{59}Fe (mμg.atoms/100mg. dry wt.)	Normal: Bound ^{59}Fe (mμg.atoms/100mg. dry wt.)	Cobalt-toxic: Total ^{59}Fe (mμg.atoms/100mg. dry wt.)	Cobalt-toxic: Bound ^{59}Fe (mμg.atoms/100mg. dry wt.)
24	64·2	10·6	558·1	45·4
30	42·7	10·5	218·7	15·2
48	38·5	9·8	85·5	7·2
54	32·3	9·3	74·6	6·3
72	30·6	9·2	53·7	5·7

Table 3. *Effect of cobalt on catalase, iron-binding compound and haem* ^{59}Fe *in the mycelia as a function of growth period in* N. crassa

The basal medium contained in 10 ml. 120·9 mμc (1 μg. of iron) as [^{59}Fe]ferric citrate. Experimental details are given in the text.

	Normal treatment				Cobalt-toxic treatment			
Period of growth (hr.)	Growth (mg. dry wt.)	Catalase activity (ml. of 10 mM-$KMnO_4$/mg. of protein/5 min.)	Iron-binding compound ($E_{440m\mu}$)	Haem ^{59}Fe (μμg.atoms/10 mg. of haemin/100 mg. dry wt.)	Growth (mg. dry wt.)	Catalase activity (ml. of 10 mM-$KMnO_4$/mg. of protein/5 min.)	Iron-binding compound ($E_{440m\mu}$)	Haem ^{59}Fe (μμg.atoms/10 mg. of haemin/100 mg. dry wt.)
24	8·2	7·2	–	74·0	3·2	8·5	0·04	81·0
48	37·0	39·2	—	91·0	19·0	10·4	0·12	73·0
72	46·2	52·3	0·03	109·9	25·2	11·0	0·22	66·0

Table 4. *Effect of cobalt on* [2-^{14}C]*glycine incorporation into the mycelial haem fraction of* N. crassa

The basal medium contained in 10 ml. 250·0 mμc (48·0 μg. of glycine) of [2-^{14}C]glycine. Experimental details are given in the text.

Treatment	Total incorporation (μmoles/g. dry wt.)	Incorporation into haem (mμmoles/10 mg. of haemin/g. dry wt.)
Normal	12·0	6·9
Cobalt-toxic	17·1	4·7

Table 5. *Effect of cobalt on* ^{59}Fe *incorporation into protoporphyrin in cell-free extracts of* N. crassa

The incubation mixture in 2 ml. contained: iron ($^{59}FeSO_4$), 24 mμmoles; cobalt ($^{60}CoCl_2$), 30 mμmoles; protoporphyrin, 30 mμmoles; tris (pH 7·8), 180 μmoles; ascorbic acid, 40 μmoles; enzyme, 0·5 ml. containing 6 mg. of protein/ml. The iron-binding fraction prepared from XFe was added in 0·1 ml. and in slight excess equivalents of the iron present in the incubation mixture. The amount of radioactivity included as ^{59}Fe or ^{60}Co was 250·0 mμc. Experimental details are given in the text.

Metal source	Metal incorporated (μμg. atoms/10 mg. of haemin)
$^{59}FeSO_4$	87·2
$^{59}FeSO_4$*	8·4
$^{59}FeSO_4$ + 30 mμmoles of $CoCl_2$	76·9
$^{59}FeSO_4$ + 60 mμmoles of $CoCl_2$	73·0
$^{59}FeSO_4$ + 150 mμmoles of $CoCl_2$	52·9
$^{59}FeSO_4$ + iron-binding fraction	257·1
$^{59}FeSO_4$ + iron-binding fraction*	98·0
$^{59}FeSO_4$ + iron-binding fraction + 30 mμmoles of $CoCl_2$	221·4
$^{59}FeSO_4$ + iron-binding fraction + 60 mμmoles of $CoCl_2$	211·3
$^{59}FeSO_4$ + iron-binding fraction + 150 mμmoles of $CoCl_2$	130·6
$^{60}CoCl_2$	9·5

* Non-enzymic incorporation (enzyme denatured or omitted).

the results are expressed on a unit mycelial weight basis. However, bound ^{59}Fe shows a significant decrease at the end of the growth period (72 hr.) in cobalt-toxic mycelia, indicating that the cobalt–iron antagonism is intracellular in this organism.

To detect the primary site of induced iron deficiency, the effects of cobalt on growth, catalase activity, iron-binding compound production, bound ^{59}Fe and haem ^{59}Fe in the mycelia were determined as a function of the growth period. From the results presented in Tables 2 and 3 it is evident that, at 24 hr. of growth in the presence of cobalt, catalase activity, bound ^{59}Fe and haem ^{59}Fe values are higher than or comparable with those of the normal mycelia. The iron-binding compound can be detected in the culture fluid even at this period of growth under cobalt-toxic conditions. The subsequent decrease in bound ^{59}Fe and haem ^{59}Fe thus indicates an effect of cobalt at the level of iron utilization for the synthesis of key metabolites.

The fall in haem ^{59}Fe values can be due to two factors, namely an effect of cobalt on the synthesis of the porphyrin moiety and a possible competition between cobalt and iron at the level of incorporation into the porphyrin nucleus. The former aspect was examined by growing the organism under cobalt-toxic conditions in the presence of [2-^{14}C]-glycine. At the end of 72 hr. growth cobalt does not inhibit total [2-^{14}C]glycine incorporation into the mycelia but inhibits incorporation into the haem fraction (Table 4).

Previously it was found that XFe can act as a good source of iron for *N. crassa* and is incorporated much faster into the mycelium than is inorganic iron of a simple chelate like ferric citrate. Also, the incorporation of inorganic iron or ferric citrate is

Table 6. *Iron incorporation from* $X^{59}Fe$ *and* $^{59}FeSO_4$ *into protoporphyrin in cell-free extracts of* N. crassa

The low specific activity of the isolated $X^{59}Fe$ necessitated the use of high concentrations of substrates. The incubation mixture in 5ml. contained: iron ($^{59}FeSO_4$ or $X^{59}Fe$), 0·5μmole; protoporphyrin, 0·6μmole; tris (pH 7·8), 360μmoles; ascorbic acid, 80μmoles; enzyme, 2ml. containing 6mg. of protein/ml. The amount of radioactivity included was 65·1mμc. Experimental details are given in the text.

Iron source	Iron incorporated (mμg. atoms/10mg. of haemin)
$^{59}FeSO_4$	12·1
$^{59}FeSO_4$*	1·3
$X^{59}Fe$	23·7
$X^{59}Fe$*	10·7

* Non-enzymic incorporation (enzyme denatured or omitted).

markedly increased when the iron-binding fraction prepared from XFe is added (Padmanaban & Sarma, 1965). The effect was therefore examined of the iron-binding fraction on iron incorporation into protoporphyrin, with and without cobalt, in cell-free extracts (Table 5). In the presence of the iron-binding fraction, ^{59}Fe incorporation into protoporphyrin is significantly enhanced as compared with when $^{59}FeSO_4$ alone is used. The superiority of this type of organic iron is further emphasized since intact $X^{59}Fe$ has been found to act as a better iron source than $^{59}FeSO_4$ (Table 6). When the organic iron is used as the iron source the non-enzymic incorporation into protoporphyrin is high and is of the same order as that of enzymic incorporation of inorganic iron. Cobalt inhibits ^{59}Fe incorporation even in the presence of the iron-binding fraction, significantly when present in definite excess of iron. However, when cobalt is used in amounts equivalent to and in place of iron, its incorporation into protoporphyrin is less than 10% of that of iron.

DISCUSSION

Increasing concentrations of cobalt result in increased production of the iron-binding compound and a corresponding fall in catalase activity (Table 1). An identical picture has been obtained when there is a decrease in the iron concentration of the medium from the optimum level (Padmanaban & Sarma, 1965). This indicates a conditioned iron deficiency brought about by cobalt.

Cobalt does not produce an iron deficiency by inhibiting the iron uptake of the organism from the medium. Instead, maximal iron incorporation into the mycelium takes place within 24hr. under cobalt-toxic conditions. However, in the mycelium, bound ^{59}Fe, haem ^{59}Fe and catalase activity show a significant decrease in cobalt toxicity at 72hr. of growth, although these parameters are either higher or not affected at 24hr. of growth as compared with the normal mycelia. Thus an intracellular manifestation of cobalt–iron antagonism is evident that probably requires the build-up of a certain concentration of cobalt in the mycelium at the competition sites. Cobalt enhances iron uptake in *Candida guilliermondii* but there is a decrease in the trichloroacetic acid-precipitable iron and the iron incorporated into the supernatant and particulate fractions (Enari, 1958).

The iron-binding compound is secreted into the medium even at 24hr. of growth in cobalt toxicity. An iron chelate similar to XFe was detected in the *N. crassa* mycelium grown under normal conditions with optimum amounts of iron (Padmanaban & Sarma, 1965). This indicates that the formation of the iron chelate in the mycelium is a very early phase in iron transport and is interfered with in cobalt toxicity, owing to the depletion of iron from the medium, leading to the secretion of the iron-binding compound into the medium before the other iron-dependent systems are affected. The mechanism of the secretion of the iron-binding compound in large amounts is not clear and some of the possibilities for this type of a phenomenon have been discussed by Neilands (1961). The formation of the iron chelate under favourable conditions can take place at the cell surface itself, since cation-binding sites are known to be located at the periphery of the cell, as for example in yeast (Rothstein & Hayes, 1956).

The intracellular cobalt–iron antagonism is evident at the level of haem synthesis. Here, cobalt inhibits the synthesis of the porphyrin moiety and in cell-free extracts inhibits iron incorporation into protoporphyrin significantly when present in definite excess of iron. Cobalt has been found to inhibit incorporation of radioactive glycine into haem by preparations of rabbit bone marrow (Laforet & Thomas, 1956). However, in *N. crassa* cobalt itself is incorporated into protoporphyrin to a poor extent even though it inhibits iron. Labbe & Hubbard (1961) have shown that the rat-liver iron–protoporphyrin chelating enzyme can utilize both iron and cobalt for the respective haem formation with equal facility. Species specificity appears to be an important factor in determining the metal specificity of these enzymes. For example, a specific cobalt–porphyrin synthase is present in *Clostridium tetanomorphum*, which exhibits very little haem-synthase activity (Porra & Ross, 1965).

The metabolic potency of $X^{59}Fe$ as a better iron source than $^{59}FeSO_4$ for haem synthesis in cell-free

extracts emphasizes the possibility that this type of organic iron formed in the cell can act as an iron donor for haem synthesis *in vivo*. Such a possibility has already been envisaged (Prelog, 1964), and it is held that, at least in micro-organisms, the sideramines, the growth-promoting compounds of the siderochromes, may play an important part in the enzymic incorporation of iron into porphyrins.

Thanks are due to Mr T. S. Satyanarayana for technical assistance. The financial assistance of the Council of Scientific and Industrial Research, New Delhi, and the Rockefeller Foundation, New York, is acknowledged.

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The Isolation and Composition of Helical Protein Microfibrils from *Hevea brasiliensis* Latex

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(*Received* 21 *May* 1965)

1. The microfibrils contained within the lutoid particles of *Hevea brasiliensis* latex obtained from young tissue have been isolated by methods based on low-speed centrifugation, isoelectric precipitation and gel filtration. 2. The isolated microfibrils behave as a single protein having an isoelectric point of about 4 as determined by paper electrophoresis. 3. The only components so far detected in the microfibrils are protein and possibly carbohydrate; nucleic acid appears to be absent. 4. The amino acid composition of the microfibril protein shows no unusual features. 5. In latex from the more mature laticiferous tissues of *H. brasiliensis*, the lutoid particles appear to be devoid of microfibrils or their protein decomposition products.

It has been known for some time that the latex of *Hevea brasiliensis* contains in suspension other particles besides rubber. Homans & van Gils (1948) showed that by low-speed centrifugation latex could be separated into two zones, an upper layer containing most of the rubber particles and a lower yellow layer or 'bottom fraction', containing a high proportion of non-rubber materials. The main component of this fraction consists of bodies named 'lutoids' by Homans & van Gils (1948). Ruinen (1950) demonstrated that the lutoids were discrete, approximately spherical particles of diameter 1–3 μ, and that the yellow colour of the bottom fraction was due to the presence of another type of particle (Frey-Wyssling particle) first described by Frey-Wyssling (1929).

Dickenson (1963), using electron microscopy, has studied in detail the contents of the latex vessel of *H. brasiliensis*. In young latex vessels, the lutoid particles were found to contain bundles of long microfibrils possessing a hollow core. Further details of the structure of the microfibrils were revealed by negative staining, the microfibrils appearing as rigid helical rods of diameter 125 Å, pitch 100 Å and with a hollow central axis of diameter 30 Å.

The present paper reports on the isolation, purification and composition of this new latex component.

MATERIALS AND METHODS

Collection of latex. Fresh *H. brasiliensis* latex was obtained from two sources: small amounts (up to 2 ml./day) were obtained by pricking the branches of 4-year-old 'illegitimate' seedlings and budded trees of clone PB 86 growing in the tropical greenhouse attached to these Laboratories. Larger amounts (25 ml./day) were obtained by cutting the stems of 4-year-old buddings (clone RRIC–6) growing in a nursery at the Rubber Research Institute of Ceylon. The latex samples were tapped into 3 vol. of ice-cold 0·11 M-tris–HCl buffer, pH 7·2, containing mannitol (0·4 M) (referred to below as 'tris–mannitol buffer').

Isolation and washing of bottom fraction. Latex samples were centrifuged at 600–800*g* for 5 min. at 0–5° to give a buff-coloured sediment with a thin layer of yellow Frey-Wyssling particles at the surface, and a white supernatant containing most of the rubber. The supernatant was discarded and much of the rubber adhering to the walls of the centrifuge tube removed with a roll of filter paper. The sediment was washed twice by resuspension in tris–mannitol buffer (5 ml./ml. of latex) and either stored at −15° or used immediately.

Extraction of proteins from bottom fraction. All extraction and purification procedures were performed at 0–5°, except separations on gel-filtration columns, which were carried out at room temperature.

Homans & van Gils (1948) had observed that the addition of water to fresh latex caused rupture of the lutoid particles owing to the decrease in osmotic pressure. Protein extracts were therefore simply prepared by stirring the washed bottom fraction with water or buffer of low osmolarity. To aid extraction, the suspensions were sometimes frozen and thawed once.

Freeze-dried bottom fraction. Freeze-dried samples of bottom fraction isolated from latex tapped from the trunks of mature trees were provided by the Rubber Research Institute of Malaya as described by Archer (1960).

Paper electrophoresis. Bottom fraction proteins were separated on Whatman no. 1 paper strips in 50 mM-veronal buffer, pH 8·6, for 16–17 hr. under a potential gradient of 5 v/cm. (Archer & Sekhar, 1955). The strips were dyed with 0·1% bromophenol blue (Kunkel & Tiselius, 1951).

Starch-gel electrophoresis. The vertical technique of Smithies (1959) was employed with a discontinuous buffer system (Poulik, 1957). Starch was purchased from the Connaught Medical Research Laboratories, Toronto, Canada. Gels were prepared in 20mM-tris–0·5mM-boric acid–6·8mM-maleic acid buffer, pH7·87, and had a water content of 13%. The anode compartment contained 0·1M-tris–50mM-maleic acid buffer, pH7·44, and the cathode compartment 50mM-boric acid–63mM-borax buffer, pH8·44. Protein samples (5mg.) were run under an initial potential gradient of 3v/cm. and a constant current of 18·5mA for 16hr. The sliced gel was stained with 0·1% Nigrosine in methanol–water–acetic acid (5:5:1, by vol.).

Spectrophotometry. Absorption spectra were determined with a Hilger Uvispek spectrophotometer.

Dextran-gel column chromatography. Sephadex G-75 (medium grade) and Sephadex CM-50 (medium grade) were purchased from AB Pharmacia, Uppsala, Sweden. The Sephadex G-75 was soaked for 24hr. in 0·11M-tris–HCl buffer, pH7·2, containing NaCl (1%, w/v), before packing. Sephadex CM-50 was washed several times with 0·5N-NaOH, 0·5N-HCl, water, 0·1M-potassium phosphate buffer, pH6·6, and finally equilibrated with 5mM-potassium phosphate buffer, pH6·6.

Amino acid analysis. Hannig's (1959) modification of the automatic procedure of Spackman, Moore & Stein (1958) was used. The sample was dried at 100° for 16hr. and then hydrolysed by heating for 24hr. at 102–103° in a sealed tube with 1000 times its weight of 6N-HCl.

Phosphorus analysis. Phosphorus was determined colorimetrically with ammonium molybdate, after wet oxidation. The samples were dried to constant weight *in vacuo* at room temperature before analysis.

Electron microscopy. The presence of microfibrils was demonstrated by using the negative staining method of Brenner & Horne (1959) as modified by Dickenson (1963). Addition of potassium phosphotungstate (pH5·3) to bottom fraction extract gave a flocculent precipitate, which was centrifuged off at low speed, and the supernatant was examined for microfibrils. Electron micrographs were prepared by using a Siemens Elmiskop I electron microscope at an instrumental magnification of 40000.

EXPERIMENTAL AND RESULTS

Detection of microfibrils by using paper electrophoresis. Dickenson (1963) had shown that the lutoid particles in mature latex vessels are devoid of microfibrils. Therefore, to see whether the microfibrils could be detected by electrophoresis, a comparison was made between the electrophoretic patterns of the proteins in bottom-fraction extracts from young and old vessels. Washed bottom fraction from 0·5ml. of young branch latex was extracted with 0·5ml. of water, the extract centrifuged at 100*g* for 3min. and the turbid supernatant used for electrophoresis. Bottom-fraction extract from mature latex vessels was prepared by stirring 50mg. of freeze-dried bottom-fraction solids in 1ml. of 10mM-sodium diethyldithiocarbamate (to inhibit enzymic oxidation of endogenous polyphenols).

The results (Fig. 1) showed that, in the latex from young branches, an extra protein component was present (band F), which was attributed to microfibrils. This conclusion was confirmed by the finding that only this component sedimented when a microfibril-containing extract was centrifuged at 175000*g* for 1hr.

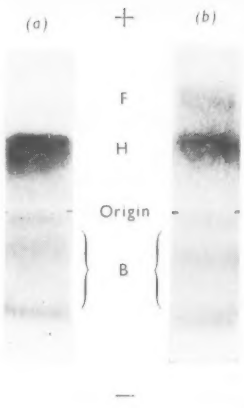
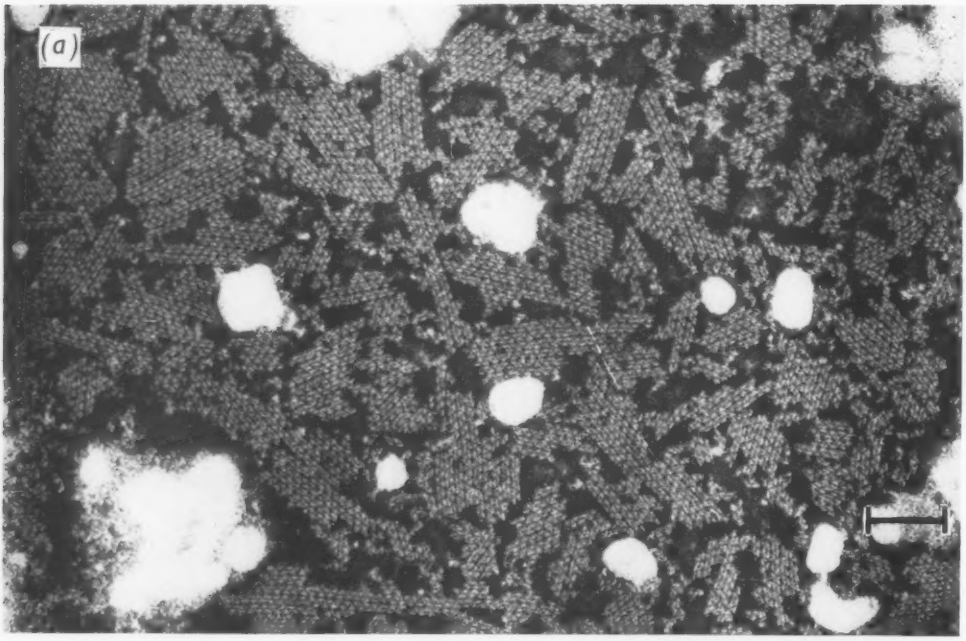


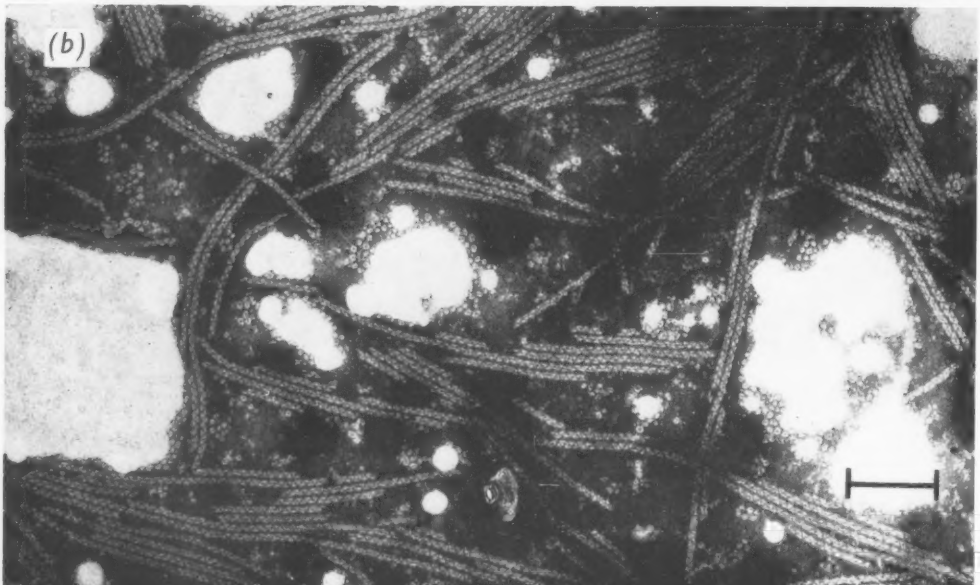
Fig. 1. Paper electrophoresis of proteins of bottom fraction from a young branch and from the trunk of a mature tree: (*a*) trunk bottom fraction; (*b*) branch bottom fraction. Electrophoresis was carried out in 50mM-veronal buffer, pH8·6, at 5v/cm. for 17hr. F, Microfibril protein; H, hevein; B, basic proteins.

Isolation of microfibrils by centrifugation (method I). Preliminary experiments showed that the microfibrils in an extract from young green branches could be sedimented readily at approx. 1700*g*, owing presumably to their aggregation. Centrifugation at much higher *g* values was less satisfactory because of extensive microfibril breakdown.

Washed bottom fraction from 2ml. samples of latex was extracted with 1ml. of water, and the extract centrifuged at 100–600*g* for 2–3min. to give maximum separation between membrane fragments and microfibrils. The residue was re-extracted with 0·4ml. of water and the turbid supernatants were pooled and centrifuged at 1750*g* for 5min. The sediment was washed twice by resuspension in 0·4ml. of water followed by centrifugation at 5000*g*. Although clear supernatants could usually be produced by this washing procedure, sedimentation of microfibrils was incomplete. After washing, the microfibrils were freeze-dried. The yield was 1mg.

Isoelectric precipitation of microfibrils (method II). The results shown in Fig. 1 indicated that the microfibrils would probably be isoelectric at a pH a little lower than 4·7, which Archer (1960) had found to be the isoelectric point of the latex protein hevein





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(band H, Fig. 1). Since hevein is readily soluble in the region of pH 4, the possibility of separating the microfibrils from hevein by isoelectric precipitation was investigated. Precipitation of microfibrils from bottom-fraction extracts proved to be maximal at pH 3·5–4·0, and paper electrophoresis showed that no significant precipitation of hevein or basic proteins occurred under these conditions.

For preparative purposes, latex from buddings of clone RRIC–6 was used. Electrophoresis established that microfibrils were present from the graft union upwards and latex was therefore collected from all parts of the stem that could be reached conveniently. Samples (25ml.) of latex were treated as follows.

A 4ml. portion of 10mM-sodium diethyldithiocarbamate was added to the washed bottom fraction and the mixture frozen and thawed once. The suspension was centrifuged at 1000*g* for 5min. and the residue re-extracted with 1ml. portions of 10mM-sodium diethyldithiocarbamate until a sample of the final extract gave no precipitate at pH 4·0. The extracts were centrifuged at 1800*g* for 5min. to remove membrane fragments and the microfibrils precipitated at pH 4·0 with acetic acid. After 1hr. at 0°, the suspension was centrifuged at 1800*g* until the supernatant was clear. The sediment was washed twice with 10mM-sodium diethyldithiocarbamate, then with acetic acid (pH 4·0) and finally with water. Plate 1(*a*) is an electron micrograph of the preparation. The material was freeze-dried and a sample examined by starch-gel electrophoresis. The remainder was dialysed exhaustively against water and freeze-dried. The yield was 8mg. The supernatant from the precipitation step was freeze-dried and stored at −15°.

Separation of bottom-fraction proteins by gel filtration. Since Archer (1960, 1963) has shown that the major non-microfibril components of bottom fraction are of low molecular weight, the possibility of purifying the microfibrils by gel filtration was examined.

Washed bottom fraction from 1ml. of branch latex was extracted with four 0·3ml. portions of water, and the combined extracts were filtered through glass wool. A 0·2ml. sample of the filtrate was applied to a column (40cm. × 1cm.) of Sephadex G-75 and the proteins were eluted with 0·11M-tris–hydrochloric acid buffer, pH 7·2; the extract was resolved into three fractions (Fig. 2). To identify the proteins, the corresponding fractions from three other gel-filtration runs were pooled, dialysed against 50mM-veronal buffer, pH 8·6, freeze-dried and examined by paper electrophoresis.

Fraction (i) (Fig. 2) contained only microfibril protein, fraction (ii) contained only basic proteins (cf. Fig. 1), and fraction (iii) contained a protein with the same mobility as hevein.

Isolation of microfibrils (method III). The washed bottom fraction from 30ml. of latex from buddings of clone RRIC–6 was suspended in 10mM-sodium diethyldithiocarbamate (5ml.) and filtered through glass wool and a no. 3 glass sinter. An equal volume of 0·2M-potassium phosphate buffer, pH 8·0, was added to the filtrate and the solution saturated with ammonium sulphate. After standing overnight at −2°, the suspension was centrifuged at 2000*g* for 10min. and the residue washed twice with neutral saturated ammonium sulphate solution. The suspension was diluted with 40ml. of 0·11M-tris–hydrochloric acid buffer, pH 7·2, containing sodium chloride (1%), filtered through a no. 3 glass sinter and the turbid filtrate chromatographed on a column (43cm. × 4cm.) of Sephadex G-75. The proteins were washed through the column with the buffer used to dilute the suspension. The separation is shown in Fig. 3. The turbid microfibril-containing fraction 1 (Fig. 3) was satu-

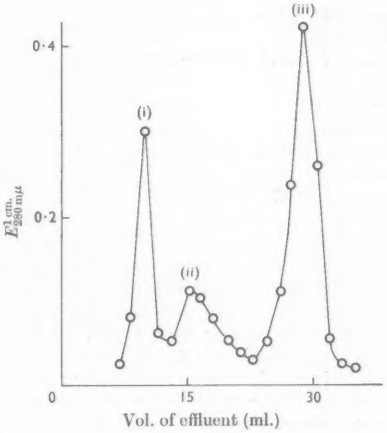

Fig. 2. Gel filtration of bottom-fraction proteins extracted from 0·2ml. of young-branch latex on a column (40cm. × 1cm.) of Sephadex G-75. The proteins were eluted with 0·11M-tris–HCl buffer, pH 7·2, containing NaCl (1%). (i) Microfibril protein; (ii) basic proteins; (iii) hevein.

EXPLANATION OF PLATE 1

(*a*) Electron micrograph of microfibrils isolated by isoelectric precipitation: negatively stained with 1% potassium phosphotungstate (magnification ×120000). (*b*) Electron micrograph of microfibrils isolated by gel filtration: negatively stained with 1% potassium phosphotungstate (magnification ×120000). The scale on each photograph indicates 1000 Å.

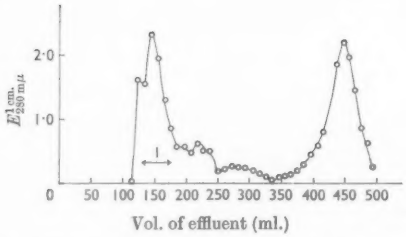

Fig. 3. Isolation of microfibril protein by gel filtration on a column (43cm. × 4cm.) of Sephadex G-75. The bottom-fraction proteins (from 30ml. of clone RRIC–6 latex) were eluted with 0·11M-tris–HCl buffer, pH7·2, containing NaCl (1%). Fraction 1 was taken for further purification.

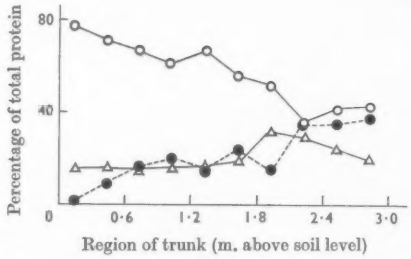

Fig. 4. Relative proportions of proteins in bottom-fraction extracts prepared from latices of increasing age. The samples, containing approx. 2mg. of protein, were analysed by gel filtration as described in the text. ●, Microfibril protein; △, basic proteins; ○, hevein.

rated with ammonium sulphate at pH7 and, after standing for 4hr. at −2°, centrifuged at 2000*g* for 15min. The sediment was washed twice with 0·11M-tris–hydrochloric acid buffer, pH7·2, saturated with ammonium sulphate, dialysed against 5mM-potassium phosphate buffer, pH6·6, and passed through a Sephadex CM-50 column (3cm. × 1cm.) equilibrated with 5mM-potassium phosphate buffer, pH6·6. The microfibril fraction, which passed straight through the column, was stored at −6°. The yield of microfibrils was 20mg. Plate 1(*b*) is an electron micrograph of this preparation.

Essentially the same gel-filtration technique was used to recover the microfibrils remaining in the supernatant after precipitation at pH4·0 (see method II).

Effect of age of laticiferous tissue on microfibril content of bottom fraction. Gel filtration was used to determine the relative proportions of the microfibrils and other major bottom-fraction proteins in latex from tissues of different age. The trunk of a 4-year-old seedling was marked out in ten consecutive intervals of 30cm., starting at soil level, and 1–2ml. samples of latex were collected from the areas between the marks. From each sample washed bottom fraction was isolated and extracted four times with water, and the extracts were freeze-dried. The freeze-dried material was dissolved in 0·11M-tris–hydrochloric acid buffer, pH7·2, containing sodium chloride (1%), the total proteins were precipitated by saturation with ammonium sulphate and the precipitate was washed with saturated ammonium sulphate solution until the $E^{1\,cm.}_{260\,m\mu}$ value of the washings was less than 0·05. A sample of the precipitate (approx. 2mg. of protein) was chromatographed on a column (40cm. × 1cm.) of Sephadex G-75. Recovery of proteins from the column was complete.

The relative amounts of microfibrils, hevein and basic proteins in the samples, determined from the areas of the respective peaks on the elution diagrams, are shown in Fig. 4.

General characteristics of the microfibrils. As often observed with asymmetric particles, suspensions of microfibrils had a 'silky' appearance when gently swirled. At a pH of about 13 the suspension became opalescent and at pH < 2 the microfibrils dissolved to give initially a clear solution. Heating the suspension (7min. at 70°) at neutral pH caused coagulation. Suspensions in 5mM-potassium phosphate buffer, pH6·6, could be stored frozen (−6°) for up to 3 months without any obvious change in their electron-microscopic appearance. Microfibrils were also stable structurally for at least 2 weeks when suspended in saturated ammonium sulphate at 0°.

Microfibrils dialysed and freeze-dried showed a less well-defined ultrastructure than those stored as frozen suspensions. This loss of definition was much less marked, however, when the pH4·0 supernatants obtained in method II were freeze-dried.

Purity of the isolated microfibrils. Microfibrils obtained by methods I and II gave single bands of the expected mobility on paper electrophoresis at pH8·6, a small amount of material remaining at the origin. Microfibrils prepared by method III behaved anomalously in that essentially all the protein remained at the origin when a suspension of the salt-free freeze-dried material was used. However, when these microfibrils were precipitated at pH4·0 and then examined electrophoretically at pH8·6, a single band with the characteristic mobility of the microfibrils was obtained. This unusual behaviour can probably be attributed to the fact that the length of the microfibrils obtained by method III was considerably greater than in the other samples.

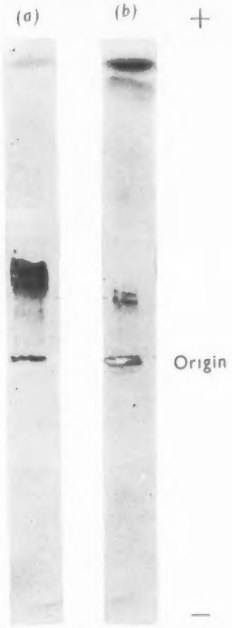

Fig. 5. Starch-gel electrophoretogram of microfibril protein purified by isoelectric precipitation (method II). For comparison, the electrophoretogram of a sample of freeze-dried supernatant solids obtained in method II is also shown. (*a*) Precipitated microfibril protein; (*b*) freeze-dried solids. Each sample was subjected to electrophoresis in the same starch-gel block for 16 hr. by using the discontinuous buffer system described in the text; 5 mg. of material was applied in each case.

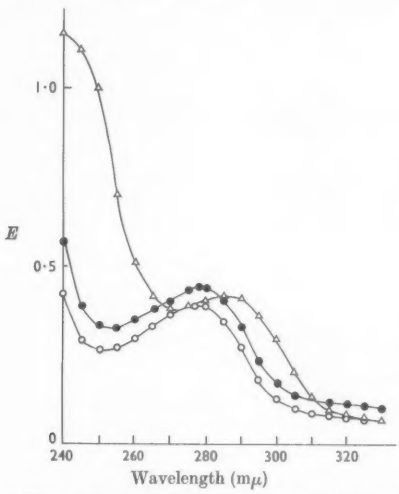

Fig. 6. Ultraviolet-absorption spectra of microfibril protein: ●, in 5 mM-potassium phosphate buffer, pH 6·6; ○, in 0·1 N-HCl; △, in 0·1 N-KOH. The spectra were measured in 1 cm. cells on solutions containing about 0·2 mg. of protein/ml.

Starch-gel electrophoresis of microfibrils (prepared by method II) showed the presence of small amounts of protein impurities (Fig. 5). The relative mobilities of the microfibrils and of hevein were the reverse of those found by paper electrophoresis, probably because the gel, acting as a molecular sieve (Smithies, 1959), hindered the movement of the microfibrils but not of hevein, which has a molecular weight of only 10000 (Archer, 1960).

Ultraviolet-aborption spectra of the isolated microfibrils. The absorption spectra of the microfibrils were characteristic of protein, but the absorption curves (Fig. 6) were considerably distorted by the strong light-scattering of the suspensions.

Attempts to correct the spectra for scattering by the method of Treiber & Schauenstein (1949) were not successful because plots of $\log\lambda$ against $\log E$ outside the region of true absorption were only approximately linear. It was not possible therefore to obtain curves sufficiently accurate for the spectroscopic determination of the tryptophan and tyrosine content of the protein.

Amino acid analysis of the isolated microfibrils. A preliminary amino acid analysis of the microfibril protein is shown in Table 1. Duplicate analyses were in good agreement, and the results given are the mean values of two determinations on a single hydrolysate. Cysteine was not determined separately as cysteic acid and the value for this amino acid in Table 1 is therefore too low. Destruction of serine and threonine was corrected for by assuming losses of 10 and 5% respectively on hydrolysis (Rees, 1946). Tryptophan was not determined. The nitrogen content of the protein was 15·3%, of which the amino acid and amide N accounted for 93·5%. Amino acid residues accounted for 87·7% of the dry weight.

Detection of carbohydrate in the isolated microfibrils. The following tests (Dische, 1955) indicated the presence of carbohydrate: (i) the microfibrils gave a cherry-red solution ($\lambda_{max.}$ 560 mμ) in the Molisch test, and a brown solution in the indole–sulphuric acid test; (ii) the absorption spectrum of the microfibrils in conc. sulphuric acid showed a maximum at 316 mμ; (iii) in the cysteine–sulphuric acid reaction, the microfibril solution had an absorption maximum at 402 mμ. The carbohydrate content estimated by the Molisch reaction with glucose as standard (Dische, 1955) was 4%.

Table 1. *Amino acid analysis of microfibril protein*

Amino acid residue	Amino acid N (g./100g. of protein N)	Amino acid residue (g./100g. of protein)	Amino acid residue (residues/1000 residues)
Arg	9·8	4·2	34
Lys	9·9	7·0	69
His	3·9	1·9	18
Glu	6·6	9·4	92
Asp	8·6	10·4	115
Thr	4·4	4·9	62
Ser	4·7	4·4	65
Gly	5·5	3·4	76
Pro	3·8	4·0	52
Tyr	4·2	7·5	59
Ala	4·8	3·7	67
Val	3·9	4·2	54
Leu	6·1	7·6	85
Ile	3·9	4·8	54
Phe	4·9	7·9	68
Met	0·8	1·1	11
½ CyS	1·2	1·3	17
NH_3	6·5	—	—
Total	93·5	87·7	

Absence of nucleic acid from the isolated microfibrils. The phosphorus content of the microfibril protein was less than 0·01%, thus showing the virtual absence of nucleic acid. This result was confirmed by extraction of the microfibril protein with aqueous phenol by the procedure of Gierer & Schramm (1956).

DISCUSSION

Both isoelectric precipitation and gel filtration (methods II and III) were satisfactory for obtaining microfibril protein in sufficient quantities for chemical characterization. Gel filtration has the advantage that yields are higher, since the losses involved in washing the microfibril suspension are avoided. These losses can be particularly serious in method I (centrifugation method), especially when very small amounts of latex are being handled. However, the centrifugation method is useful when small samples only are required, e.g. for electron microscopy. The paper and starch-gel electrophoresis results indicate that all three methods of isolation yield microfibril protein of about the same degree of purity.

Although the amino acid analysis was only of a preliminary nature, the high weight and nitrogen recoveries obtained indicate that the microfibrils are probably composed entirely of protein. With the exception of tryptophan, for which no data are available, the microfibrils contain all the amino acids commonly found in proteins, and the amino acid composition itself does not possess any unusual features. At one stage of this investigation it was thought that the microfibrils might be built up from sub-units consisting of hevein. Comparison of the amino acid analysis of the microfibril protein with that of hevein (Archer, 1960) rules out this possibility. Besides protein, the only other material detected in the microfibril preparations was carbohydrate, which may, however, be present as an impurity.

Gel-filtration analyses and paper-electrophoretic examination of extracts of bottom fraction from latices drawn from tissues of different age has confirmed Dickenson's (1963) observation that lutoid particles in sufficiently old latex vessels are devoid of microfibrils. Only basic proteins and hevein were found in extracts of bottom fraction prepared from the latex of old tissue, hevein comprising about 80% of the total protein in such samples. The marked differences between the amino acid composition of hevein and the microfibrils and the fact that hevein has been found in substantial amounts in bottom fraction from very young laticiferous tissue makes it most unlikely that this protein is an end product of microfibril decomposition. Thus it appears that, as the lutoid particles age, the microfibrils disappear without being converted into other proteins that remain in the particle, indicating that the lutoid particles may well be a site of proteolytic activity. The disappearance of the microfibrils suggests that they may provide a store of protein that is utilized by the developing tissue. However, this concept of their role does not take into account their highly orientated helical structure. It seems more likely that the microfibrils have a more specific function that may become apparent when further information is available about the role of the lutoid particles themselves in the metabolism of the latex vessel.

This work forms part of a programme of research undertaken by the Natural Rubber Producers' Research Association. The author thanks Dr E. D. C. Baptiste and Mr C. A. de Silva of the Rubber Research Institute of Ceylon for provision of facilities at the Institute, and their Staff for help in the collection and processing of latex. I also thank the British Leather Manufacturers' Research Association for the amino acid analysis, Mr M. Stubbs for electron micrographs, Dr E. G. Cockbain, Dr B. L. Archer and Dr P. B. Dickenson for helpful discussion, and Miss C. M. Hepper for technical assistance.

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Changes in Lipid Composition of the Maturing Rat Testis

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(*Received* 12 *July* 1965)

1. The lipids and fatty acids of the lipids of testes of rats aged 4 weeks to 6 months were separated and analysed. 2. A decrease in concentration of triglyceride was noted, but there was no significant change in the concentration of phospholipids, plasmalogen or cholesterol during this time. 3. There were no significant differences in the total lipid concentration of palmitic acid, stearic acid, linoleic acid, arachidonic acid and docosatetraenoic acid between the various age groups. 4. A decrease in the concentration of oleic acid in the phosphatide and triglyceride fractions and an increase in the concentration of docosapentaenoic acid (characterized as the $\Delta 4,7,10,13,16$-isomer) in phosphatides but not in triglyceride were observed during the maturation period. 5. Histological studies indicated that the lipid changes occurred at the same time as the appearance and maturation of the spermatids.

The increased concentration of pentaenoic acid in testicular tissue of rats with age (3 weeks to 3 months) observed by Kirschman & Coniglio (1961) suggests that lipids may have an essential role in the maturation of the testis. Although several reports of lipid composition of testicular tissue are available, these contain only partial analyses of lipids and fatty acids and were done in rats of only one age group. Thus Migeon (1952) determined lipid phosphorus and cholesterol in testes of intact and adrenalectomized rats given cortisone. Aaes-Jorgensen (1958) studied the effects of conjugated isomers of dienoic and trienoic fatty acids on the polyunsaturated fatty acid composition of rat testicular lipids as determined by the alkaline isomerization method. Analyses of testicular phospholipids were reported by Collins & Shotlander (1961) and by Scott, Dawson & Rolands (1963). The fatty acids of phospholipids and triglycerides of rat testis were determined by Paoletti & Grossi (1961), but their data did not include pentaenoic acids. In a more recent study Bieri & Andrews (1964) reported results of the effects of vitamin E deficiency on the fatty acids of total lipid and of phospholipid of rat testis.

The purpose of the present investigation was to determine in detail the lipid composition of testicular tissue of rats of various ages and to attempt to correlate the chemical findings with changes observed in the morphology of the organ.

EXPERIMENTAL

Animals. Sprague–Dawley rats from our own colony were maintained on Purina laboratory chow after weaning (21 days). The testes of a number of animals from each age group (4, 6, 7, 9, 13 and 29 weeks) were pooled for fractionation of lipids. Total fatty acid patterns were done in most cases on organs of individual animals.

Extraction and fractionation procedures. Testes were removed from animals immediately after decapitation and homogenized in a Waring Blendor with 20vol. of chloroform–methanol (2:1, v/v) for 3min. The homogenate was allowed to stand at room temperature for at least 30min. before filtration. The residue was re-extracted twice in the same manner. The combined filtrates were evaporated to dryness in a rotary evaporator at room temperature and the lipid residue was dissolved in chloroform. A portion was removed for chemical analyses, and the remainder was chromatographed on an 18g. silicic acid column according to the method of Hirsch & Ahrens (1958). Batch elutions were collected and evaporated to dryness in a rotary evaporator. The lipid residue was then dissolved in chloroform. When phospholipid was present, 5% (v/v) of methanol was added to the chloroform. A portion was removed for chemical analyses and thin-layer chromatography and the remainder was used for analysis of fatty acids. The phospholipid fraction was chromatographed on DEAE-cellulose according to the method of Rouser, Kritchevsky, Heller & Lieber (1963). Neutral lipids were separated by thin-layer chromatography with light petroleum (b.p. 40–60°)–diethyl ether–acetic acid (85:15:1, by vol.) and phospholipids with chloroform–methanol–water (95:35:4, by vol.). Standards for thin-layer chromatography were obtained from Applied Science Laboratories, State College, Pa., U.S.A.

Analytical procedures. The following analyses were performed on samples of the appropriate fractions: total weight, fatty acid ester (Rapport & Alonzo, 1955), phosphorus (King, 1932), cholesterol (Sperry & Webb, 1950) and plasmalogen (Gottfried & Rapport, 1962).

Extraction and methylation of fatty acids. Fatty acids were obtained by extraction with light petroleum of the

saponified and acidified fractions. Methyl esters were prepared by the method of Metcalf & Schmitz (1961).

Gas–liquid chromatography. Gas–liquid chromatography of the methyl esters of fatty acids of total lipid, triglyceride and phospholipid was done with a Barber–Colman model 10 instrument equipped with an argon ionization detector. The column was 6ft. × 4mm. internal diam. and was packed with diethylene glycol succinate polyester (12·0%, by wt.) coated on 110–120 mesh chromosorb P. Column temperature was 197° and gas-flow rate 140–150ml./min. The detector was calibrated with pure methyl esters of fatty acids (including arachidonic acid and docosahexaenoic acid) obtained from the Hormel Institute, Austin, Minn., U.S.A., Applied Science Laboratories, and National Institutes of Health, Bethesda, Md., U.S.A.

Fatty acids were identified by the following criteria: the number of carbon atoms was determined by hydrogenating and comparing the saturated fatty acid with pure standards by gas–liquid chromatography; the degree of unsaturation was determined by alkaline isomerization (Holman, 1957); the positions of the double bonds were determined by oxidation with periodate–permanganate by the method of Rudloff (1956) and gas–liquid-chromatographic analyses of the fragments.

Light-microscopy. Histological examination was done on a transverse section of tissue taken through the midpoint perpendicular to the long axis and fixed in Helly's solution. The section was dehydrated with dioxan and acetone and stained with PAS–Harris haematoxylin (Leblond & Clermont, 1952*a*). Spermatogenesis has been classified according to the system of Leblond & Clermont (1952*b*).

Materials. All solvents were reagent grade except light petroleum, which was AOCS grade, redistilled and passed over activated silicic acid. Silicic acid for column chromatography was obtained from Bio-Rad Laboratories, Richmond, Calif., U.S.A., and silica gel G for thin-layer chromatography was obtained from Research Specialties Inc., Berkeley, Calif., U.S.A. Boron trifluoride–methanol reagent used for methylation of fatty acids was obtained from Applied Science Laboratories.

RESULTS

The concentration of various lipids in the testis of the maturing rat is given in Table 1. There was no significant change in the concentration of total lipid, cholesterol, phospholipid and plasmalogen with age. However, there was a significant decrease in the concentration of triglyceride (4 to 13 weeks, $P < 0.001$) and of sterol ester (4 to 13 weeks, $P < 0.02$). In all age groups 2·38–2·64% of the wet weight of the testes was lipid, and of this the largest component (about 60–70%) was phospholipid. Unesterified cholesterol and triglyceride were present in smaller concentrations, and cholesterol ester and diglyceride were found only in small quantities. The diglyceride may have been formed by deacylation of the triglyceride during isolation of the lipids. The presence of a small amount of free fatty acids contaminating the cholesterol fraction supports the possibility of deacylation.

The phospholipids were separated into nine fractions by DEAE-cellulose column chromatography. Phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and sphingomyelin

Table 1. *Concentration of lipids in testis of the maturing rat*

Results are given as mean values ± S.E.M. (number of analyses in parentheses). Each analysis was made on a pool of organs from four to six animals.

Age (weeks)	Total lipid (mg./g.)	Concentration (μmoles/g. wet wt. of tissue)					
		Sterol ester	Triglyceride	Cholesterol	Diglyceride	Lipid phosphorus	Plasmalogen
4	26·4±0·01 (3)	0·25±0·02 (3)	2·9±0·1 (3)	4·7±1·0 (3)	0·33±0·02 (3)	16·6±0·3 (3)	1·80±0·16 (3)
6	23·8±1·32 (3)	0·18±0·03 (3)	2·0±0·1 (3)	5·1±0·2 (3)	0·30±0·02 (3)	20·5±2·1 (3)	2·33±0·31 (3)
7	25·4±0·01 (2)	0·16±0·05 (2)	1·2±0·2 (2)	4·0±0·2 (2)	0·23±0·02 (2)	16·6±0·1 (2)	2·05±0·04 (2)
9	25·4±2·30 (3)	0·14±0·02 (3)	1·0±0·1 (3)	4·7±0·2 (3)	0·19±0·02 (3)	17·8±2·0 (3)	2·27±0·18 (3)
13	26·1±0·03 (3)	0·15±0·01 (3)	1·4±0·3 (3)	5·8±1·0 (3)	0·21±0·005 (3)	14·1±2·3 (3)	2·10±0·34 (3)
29	24·2±0·05 (4)	0·12±0·01 (4)	1·0±0·1 (4)	4·6±0·1 (4)	0·24±0·005 (4)	16·6±0·3 (4)	2·38±0·07 (4)

Table 2. *Major phosphatides of rat testicular lipid*

	Lipid phosphorus (μmoles/g.)			Lipid phosphorus (moles/100 moles)		
Age (weeks).....	4	6	13	4	6	13
Choline	6·27	8·43	7·22	37·8	50·7	44·6
Ethanolamine	2·77	5·41	4·17	16·7	32·6	25·8
Serine	1·30	0·52	0·90	7·9	3·1	5·6
Sphingomyelin	0·15	0·99	1·01	0·9	6·0	6·3

Table 3. *Major fatty acids of total lipid of testis*

Results are given as mean values ± S.E.M. (numbers of analyses in parentheses).

Age (weeks)	Concentration (mg./g. wet wt. of tissue)						
	$C_{16:0}$ acid	$C_{18:0}$ acid	$C_{18:1}$ acid	$C_{18:2}$ acid	$C_{20:4}$ acid	$C_{22:4}$ acid	$C_{22:5}$ acid
4	3·59 ± 0·34 (6)	0·67 ± 0·10 (6)	2·06 ± 0·11 (6)	0·48 ± 0·05 (6)	2·21 ± 0·17 (6)	0·17 ± 0·02 (6)	0·83 ± 0·09 (6)
5	3·65 ± 0·17 (4)	0·60 ± 0·03 (4)	1·58 ± 0·11 (4)	0·45 ± 0·06 (4)	2·17 ± 0·11 (4)	0·16 ± 0·03 (4)	1·13 ± 0·05 (4)
6	3·90 ± 0·09 (6)	0·65 ± 0·02 (6)	1·75 ± 0·04 (6)	0·63 ± 0·03 (6)	1·89 ± 0·12 (6)	0·18 ± 0·03 (6)	1·32 ± 0·06 (6)
7	3·63 ± 0·18 (5)	0·58 ± 0·04 (5)	1·43 ± 0·06 (5)	0·61 ± 0·02 (5)	2·11 ± 0·13 (5)	0·17 ± 0·03 (5)	1·81 ± 0·09 (5)
9	3·64 ± 0·09 (3)	0·48 ± 0·02 (3)	1·41 ± 0·02 (3)	0·63 ± 0·05 (3)	1·87 ± 0·14 (3)	0·23 ± 0·04 (3)	2·09 ± 0·18 (3)
13	3·76 ± 0·27 (3)	0·54 ± 0·02 (3)	1·33 ± 0·08 (3)	0·63 ± 0·05 (3)	1·84 ± 0·05 (3)	0·23 ± 0·03 (3)	1·99 ± 0·13 (3)
29	3·69 ± 0·10 (4)	0·50 ± 0·03 (4)	1·38 ± 0·10 (4)	0·62 ± 0·04 (4)	1·83 ± 0·09 (4)	0·19 ± 0·02 (4)	2·00 ± 0·09 (4)

Table 4. *Characterization of the testicular unsaturated fatty acids*

Relative retention*	Product of hydrogenation	Highest wavelength of u.v.-absorption peaks after alkaline isomerization	Major products of oxidation		Designation
			Monocarboxylic acid	Dicarboxylic acid	
0·68	$C_{16:0}$ acid	None	Heptylic acid	Azelaic acid	Δ^9-$C_{16:1}$ acid
1·16	$C_{18:0}$ acid	None	Pelargonic acid	Azelaic acid	Δ^9-$C_{18:1}$ acid
1·49	$C_{18:0}$ acid	233 mμ	Hexanoic acid	Azelaic acid	$\Delta^{9,12}$-$C_{18:2}$ acid
3·08	$C_{20:0}$ acid	268 mμ	Hexanoic acid	Suberic acid	$\Delta^{8,11,14}$-$C_{20:3}$ acid
3·52	$C_{20:0}$ acid	315 mμ	Hexanoic acid	Glutaric acid	$\Delta^{5,8,11,14}$-$C_{20:4}$ acid
6·24	$C_{22:0}$ acid	315 mμ	Hexanoic acid	Pimelic acid	$\Delta^{7,10,13,16}$-$C_{22:4}$ acid
7·22	$C_{22:0}$ acid	346 mμ	Hexanoic acid	Succinic acid	$\Delta^{4,7,10,13,16}$-$C_{22:5}$ acid
9·60	$C_{22:0}$ acid	375 mμ	—	—	$C_{22:6}$ acid
10·79	$C_{24:0}$ acid	315 mμ	—	—	$C_{24:4}$ acid
12·13	$C_{24:0}$ acid	346 mμ	—	—	$C_{24:5}$ acid

* Retention of methyl ester relative to that of methyl stearate by gas–liquid chromatography.

were the most abundant phosphatides present (Table 2). Small amounts of inositol phosphatides, lysophosphatidylethanolamine, phosphatidic acid and polyglycerol phosphatides were also shown to be present.

The fatty acid composition of the total lipids for the various ages is shown in Table 3. There was no significant difference in the concentrations of palmitic acid, stearic acid, linoleic acid, arachidonic acid and docosatetraenoic acid with age. On the other hand, there was a significant decrease in concentration with increasing age of the oleic acid (4 to 13 weeks, $P < 0.005$) and a significant increase in concentration with age of the docosapentaenoic acid (4 to 13 weeks, $P < 0.001$). In addition to the fatty acids shown in Table 3 small amounts (less than 1%) of $C_{12:0}$ acid, $C_{14:0}$ acid, $C_{15:0}$ acid, $C_{16:1}$ acid, $C_{17:0}$ acid, $C_{20:0}$ acid, $C_{20:1}$ acid, $C_{20:3}$ acid (1–2%), $C_{22:6}$ acid, $C_{24:4}$ acid and $C_{24:5}$ acid have been detected. The results of the structural determinations of the unsaturated fatty acids are summarized in Table 4. All of these fatty acids except the $C_{24:5}$ acid were reported by Holman & Hofstetter (1965) to be present in bovine and porcine testes. The $C_{24:5}$ acid, which we believe has not been reported before, was characterized only with respect to chain length and number of double bonds.

In Table 5 is summarized the major fatty acid composition of the phospholipid and triglyceride fractions. These data show the same increase in pentaenoic acid concentration in phosphatides (but not in triglycerides) as occurred in total lipid. A significant decrease was observed in the concentration of stearic acid and oleic acid in both the phosphatides and triglycerides.

The histological studies showed that very few tubules of 4-week-old rats contained young spermatids. After 5 weeks spermatids were seen in all tubules. At 7 weeks maturing spermatids were present in all tubules. These observations agree with those reported by Clermont & Perey (1957).

DISCUSSION

The most consistent chemical changes found in the lipids of maturing testes were: (1) a decrease in

Table 5. *Major fatty acids of phospholipids and triglycerides of rat testes*

Results are given as mean values ±S.E.M. (numbers of analyses in parentheses). Each sample consisted of organs pooled from four to six animals.

Age (weeks)	$C_{16:0}$ acid	$C_{18:0}$ acid	$C_{18:1}$ acid	$C_{18:2}$ acid	$C_{20:4}$ acid	$C_{22:4}$ acid	$C_{22:5}$ acid
	Concentration (mg./100g. wet wt. of tissue)						
	Phospholipid						
4	303± 2 (3)	62±1 (3)	163± 1 (3)	28±4 (3)	189± 5 (3)	11±2 (3)	80± 8 (3)
6	384±32 (3)	66±9 (3)	158±11 (3)	50±5 (3)	220±27 (3)	13±5 (3)	130±14 (3)
7	302± 2 (2)	53±1 (2)	122± 1 (2)	42±1 (2)	175± 7 (2)	12±1 (2)	150± 1 (2)
9	305±12 (3)	59±4 (3)	114± 8 (3)	36±2 (3)	206±25 (3)	13±7 (3)	173±22 (3)
13	243±47 (3)	39±9 (3)	82± 9 (3)	25±6 (3)	150±24 (3)	10±1 (3)	174±12 (3)
29	281±13 (4)	43±3 (4)	101± 1 (4)	39±1 (4)	182± 5 (4)	10±1 (4)	179± 4 (4)
	Triglyceride						
4	60·0±0·9 (3)	9·3±0·6 (3)	85·0±3·3 (3)	46·7±1·9 (3)	4·3±0·3 (3)	2·7±0·3 (3)	14·3±0·3 (3)
6	53·7±3·4 (3)	5·3±0·6 (3)	45·3±5·8 (3)	24·7±2·9 (3)	3·3±0·6 (3)	3·7±0·7 (3)	12·3±2·7 (3)
7	32·0±3·4 (2)	3·5±1·0 (2)	28·0±4·9 (2)	15·0±2·0 (2)	3·5±1·0 (2)	2·5±0·1 (2)	14·5±1·8 (2)
9	28·0±3·3 (3)	1·7±0·1 (3)	19·3±1·0 (3)	14·0±1·0 (3)	2·3±0·3 (3)	2·3±0·3 (3)	12·3±1·5 (3)
13	33·0±3·8 (3)	2·0±0·2 (3)	25·7±5·1 (3)	13·3±2·3 (3)	3·7±0·6 (3)	4·0±0·7 (3)	20·0±2·1 (3)
29	24·3±1·2 (4)	1·8±0·3 (4)	20·0±1·9 (4)	9·5±0·5 (4)	2·5±0·3 (4)	2·5±0·2 (4)	14·5±1·0 (4)

triglyceride concentration; (2) a decrease in concentration of stearic acid and of oleic acid in triglycerides; (3) a decrease in concentration of oleic acid in phospholipids; (4) an increase in concentration of the docosa-4,7,10,13,16-pentaenoic acid in phospholipids. These chemical changes occurred for the most part between 4 and 7 weeks. This was the period when the testis was undergoing maturation as evidenced from the morphological studies presented here and from the work of Clermont & Perey (1957).

Several phenomena were occurring during this 3-week period: (1) the Sertoli cells matured (Clermont & Perey, 1957); (2) the Leydig cells increased their activity (Roosen-Runge & Anderson, 1959); (3) the spermatids appeared and matured into spermatozoa. The chemical changes that occurred could have been associated with any or all of these morphological changes, or any one chemical change could have been associated with any one of the morphological changes. Further studies (unilateral cryptorchidism, cadmium toxicity and treatment with antispermatogenic drugs) indicate that the increase in concentration of the docosa-4,7,10,13,16-pentaenoic acid was associated with the appearance and maturation of the spermatids. Further evidence that the lipid changes are associated with the appearance and development of spermatids was obtained in one analysis of pooled rat epididymal spermatozoa. The concentrations of the major fatty acids ($C_{16:0}$ acid, 26·3%; $C_{20:4}$ acid, 17·9%; $C_{22:5}$ acid, 16·4%) were similar to those of the total testis. Since the spermatids account for more than 60% of the volume of the tissue of testis (Roosen-Runge, 1956), the lipids of the spermatids would be expected to dominate the lipid pattern of the whole organ.

Since many new structures develop in the maturing testis, the need for biosynthesized lipid increases. The 'lipid cycle', described by many authors (Ebner, 1888; Lynch & Scott, 1951; Smith & Lacy, 1959; Lacy, 1960, 1962), may represent a cycle of synthesis and utilization. This process is apparently associated with the embedding and releasing of the spermatids from the cytoplasm of the Sertoli cells. When the spermatids are embedded in the Sertoli cytoplasm, the lipid moves to surround the head of the spermatid and is apparently taken up by the spermatid. A particular lipid taken up at this time may be a docosapentaenoic acid-rich lipid needed for particular structures. The increase in concentration of this acid in the maturing rat has been observed in testis only.

Our data suggest the possibility that the pentaenoic acid was replacing the stearic acid and oleic acid in the phospholipid and triglyceride molecules. With a decrease in the concentration of triglyceride/g. of tissue, there was expected a decrease in the concentration of fatty acids contributed by this fraction. This proved to be the case for all fatty acids except docosatetraenoic acid and docosapentaenoic acid, which remained constant. Further, the decrease in concentrations of oleic acid and stearic acid, but not of other fatty acids, was greater during the period 4–9 weeks than the decrease in concentration of trigylcerides during the same time.

Marquis & Fritz (1965) have published evidence

of increased concentrations of carnitine and carnitine acetyltransferase in tissues of the reproductive tract of rats during the period of sexual maturation. They further noted that spermatozoa had the highest specific activities of transferases thus far observed in tissues. These observations furnish additional evidence of increased lipid metabolism in rat testis during the period of growth associated with rapid differentiation and formation of spermatids.

Holman & Greenberg (1953) suggested that the large quantity of polyunsaturated fatty acids in gonadal tissue may have some biochemical significance. This significance may lie, for the rat at least, in the fact that these polyunsaturated fatty acids belong to the linoleic acid series and possess essential fatty acid properties (DeIongh & Thomasson, 1956; Verdino, Blank, Privett & Lundberg, 1964; Davis, Bridges & Coniglio, 1965). Replacement of stearic acid and oleic acids by docosa-4,7,10,13,16-pentaenoic acid, a member of the linoleate series, at the time of maximum development of the testis and of spermatids lends further evidence to the importance of the linoleic acid family in the specific functions of tissues.

This work was supported by Grant no. AM06483 from the U.S. Public Health Service. We thank Dr G. H. Barney, Department of Pathology, for kind assistance in the preparation of the histological material.

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Purification and Properties of Galactokinase from Pig Liver

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(*Received* 19 *July* 1965)

1. Galactokinase has been purified from the liver of young pigs by high-speed centrifugation, chromatography on Sephadex G-100 and DEAE-cellulose, and ammonium sulphate fractionation. 2. The enzyme preparation has a specific activity of 10–18 μmoles of galactose phosphorylated/mg. of protein/min. at 37° and has been purified 400-fold from the liver supernatant. 3. Purified liver galactokinase has Michaelis constants of 1×10^{-4}–3×10^{-4}M for galactose and 2×10^{-4}M for ATP–Mg^{2+}, and the enzyme reaction produces equimolar amounts of galactose 1-phosphate and ADP. 4. Galactokinase phosphorylates 2-deoxygalactose and galactosamine in addition to galactose, has a pH optimum of 7·8, a Q_{10} of 2, and is stimulated by cysteine and other thiols. 5. With the exception of substrate specificity, the properties of liver galactokinase are similar to galactokinase purified from yeast and *Escherichia coli*.

Kosterlitz (1943) isolated galactose 1-phosphate from the liver of rabbits that had been fed with galactose and on the basis of this result he postulated the existence of galactokinase (EC 2.7.1.6). The enzyme was detected in yeast (Caputto, Leloir & Trucco, 1948; Wilkinson, 1949) in liver and brain (Cardini & Leloir, 1953), in erythrocytes (Schwartz, Goldberg, Komrower & Holzel, 1956) and in *Escherichia coli* (Cardini, 1951). The enzyme catalyses the following reaction:

$$\text{ATP} + \text{galactose} \rightleftharpoons \text{ADP} + \text{galactose 1-phosphate}$$

Galactokinase has been partially purified from *E. coli* (Sherman & Adler, 1963) and from yeast (Heinrich, 1964).

In the present paper a 400-fold purification of galactokinase from young pig liver is described, and some of the properties of the purified enzyme are disclosed.

MATERIALS AND METHODS

Chemicals. ATP, ADP, NADH, phosphoenolpyruvate, *N*-acetyl-D-galactosamine, 6-deoxy-D-galactose, 6-deoxy-L-galactose, galactose 6-phosphate, galactose 1-phosphate and DEAE-cellulose (capacity 0·9 mg./g., fine mesh) were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Pyruvate kinase, lactate dehydrogenase, 2-deoxygalactose, 2-deoxyglucose, galactosamine and dithiothreitol were purchased from Calbiochem, Los Angeles, Calif., U.S.A. Amberlite CG-400 (100–200 mesh) was obtained from Mallinckrodt Chemical Works, New York, N.Y., U.S.A.; Sephadex G-100 and G-200 (bead forms) were from Pharmacia, Uppsala, Sweden. [1-^{14}C]Galactose was from The Radiochemical Centre, Amersham, Bucks.

Radioactivity. Measurements were made with a Packard scintillation detector (model 314EX) with the solvent system of Herberg (1960).

Protein. Protein was measured by the u.v. method of Warburg & Christian (1941).

Galactokinase assay. ADP formed in the galactokinase reaction from ATP and galactose was measured spectrophotometrically at 340mμ with phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase and NADH by the rate of oxidation of NADH. The assay is similar to that used by Heinrich (1964). Each cuvette contained 300 μmoles of triethanolamine (adjusted to pH 7·5 with 2N-HCl), 100 μmoles of KCl, 15 μmoles of cysteine hydrochloride (adjusted to pH 7 with 2N-NaOH), 1 unit each of pyruvate kinase and lactate dehydrogenase, 1 μmole of NADH, 2 μmoles of phosphoenolpyruvate, 2 μmoles of galactose, 2 μmoles of ATP (mixed with 2 μmoles of $MgCl_2$ and adjusted to pH 7·5 before addition) and enzyme. The total volume was 3·0 ml. and the reaction was measured at 37°; 50 μmoles of NaF were also added to the reaction mixture in all assays before DEAE-cellulose chromatography to inhibit an adenosine-triphosphatase activity in the extract. This activity was separated during Sephadex chromatography. In these assays the galactokinase activity was obtained by subtracting the blank activity in the absence of galactose. When this correction was made the extinction change was linear with time for at least 10 min. and proportional to the amount of enzyme preparation added. One unit of galactokinase activity is defined as the amount of enzyme that will catalyse the formation of 1 μmole of ADP/min. at 37°.

The assay shows a complete requirement for galactose, lactate dehydrogenase, pyruvate kinase, phosphoenolpyruvate, ATP, $MgCl_2$ and enzyme. The enzyme activity was stimulated by cysteine (see Table 2) and slightly increased by KCl. Assays of galactokinase with 1, 2 and 3 μg. of purified enzyme show linearity of reaction rate with enzyme concentration (Fig. 1).

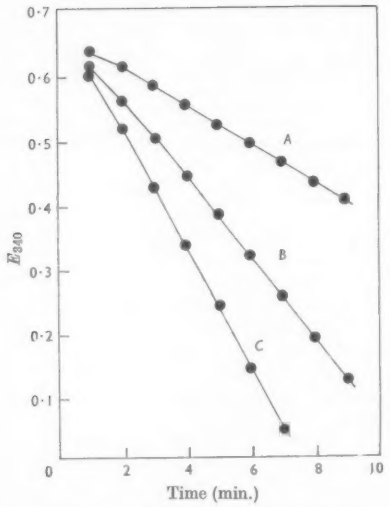

Fig. 1. Assay of galactokinase with the purified enzyme. The extinction change at 340mμ was followed spectrophotometrically. The extinction changes were 0·032, 0·063 and 0·095/min. for 1, 2 and 3μg. of enzyme (curves *A*, *B* and *C* respectively).

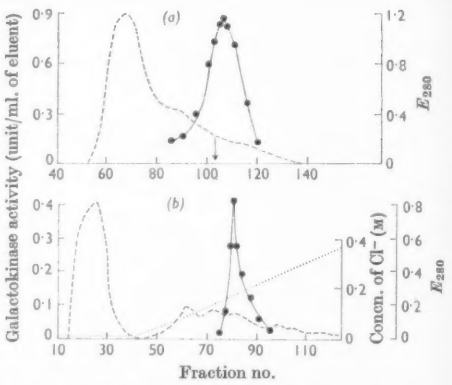

Fig. 2. (*a*) Chromatography of a pig-liver extract on a Sephadex G-100 column. The extinction at 280mμ (----) and the activity of galactokinase (●—●) are indicated. The arrow shows the elution volume of haemoglobin as determined by the extinction at 412mμ. (*b*) Chromatography of the eluate from Sephadex G-100 on DEAE-cellulose. The extinction at 280mμ (----), the activity of galactokinase (●—●) and the concentration of chloride (····) are indicated. In both experiments the fraction volume was 4·5ml. Additional experimental details are given under steps (3) and (4) of the purification procedure.

RESULTS

Purification of galactokinase

(1) *Choice of animals.* Preliminary experiments indicated that the activity of galactokinase was very low and difficult to measure in liver from adult rats. Both young rats and young pigs have higher liver galactokinase activity but pigs were chosen as more tissue could be obtained. The pigs (2 weeks old) were killed within 5hr. of the time they were taken from the sow.

(2) *Preparation of liver extract.* A pig was killed by decapitation and the liver placed in a solution containing: tris (50mM), potassium chloride (150mM), magnesium chloride (5mM) and β-mercaptoethanol (10mM) (adjusted to pH7·5 with 2N-hydrochloric acid) at 4°. All the following steps in the purification were carried out at 0–4°. A 50g. portion of liver was homogenized in 50ml. of the above solution with coaxial homogenizers. The homogenate was centrifuged at 105000*g* for 1hr. in a Spinco model L centrifuge. The supernatant (volume approx. 40ml.) was fraction I.

(3) *Chromatography on Sephadex G-100.* The Sephadex was suspended in a solution (solution A) containing: tris (20mM), galactose (1mM), EDTA (4mM), magnesium chloride (5mM) and β-mercaptoethanol (10mM) (adjusted to pH7·5 with 2N-hydrochloric acid) and poured into a column as described by Flodin (1961) to give a bed of 16cm.^{2} × 45cm. At least 1l. of solution A was passed through the column before the sample was applied. The supernatant from step (2) was passed on to the column and washed into the resin bed with solution A as described by Flodin (1961). The column was eluted with solution A, 5ml. fractions being collected at a flow rate of 1·5–2ml./min. Those fractions containing galactokinase activity (Fig. 2) were pooled to give fraction II. Sephadex G-200 may be used for this step but Sephadex G-100 is preferred since the flow rate is greater.

(4) *Chromatography on DEAE-cellulose.* The resin was suspended in solution A and poured in the same way as for Sephadex to give a column with bed dimensions 4·9cm.^{2} × 25cm. No pressure was applied to the column either in the preparation or in the chromatography. The column was washed with 1l. of 10mM-EDTA and then 1l. of solution A. The combined fractions from Sephadex G-100 chromatography were applied to the column and washed into the DEAE-cellulose with 100ml. of solution A. The galactokinase was eluted with an approximately linear gradient of solution A containing 0–1M-potassium chloride at a flow rate of 1·5–2ml./min. The gradient volume was 700ml. The enzyme was eluted between 0·2M- and 0·3M-

Table 1. *Purification of galactokinase from pig liver*

A summary of the purification procedure for galactokinase from 50g. of pig liver is shown. The details of the assay of galactokinase are given in the Materials and Methods section.

Fraction	Volume (ml.)	Total activity (units)	Total protein (mg.)	Specific activity (units/mg. of protein)	Purification factor	Recovery (%)
I: 105000*g* supernatant from pig liver	38	50·3	1420	0·035	1	100
II: combined fractions from Sephadex G-100	55	28·4	127	0·22	6·4	56
III: combined fractions from DEAE-cellulose	48	9·6	6·9	1·4	40	19
VI: 40–60% saturation $(NH_4)_2SO_4$ precipitate	2	7·6	0·5	15·2	435	15

Table 2. *Effect of thiols on galactokinase activity*

Enzyme activity was measured by ADP formation as described in the Materials and Methods section, except that cysteine was omitted and replaced by the additions mentioned below.

Addition	Concn. (mM)	Relative activity
None	–	1·0
Cysteine	5	3·4
	1	2·4
Glutathione	5	2·9
	1	2·3
β-Mercaptoethanol	5	2·8
	1	1·1
Ethanethiol	5	1·4
	1	1·1
Dithiothreitol	5	2·4
	1	1·9

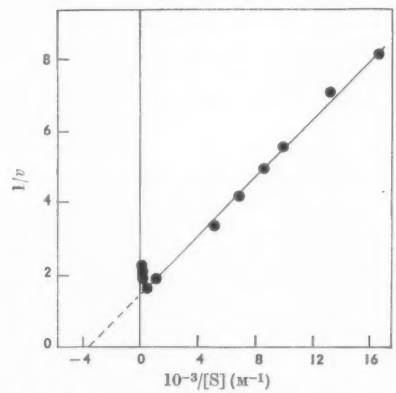

Fig. 3. Effect of galactose concentration on the activity of galactokinase. The data are plotted by the method of Lineweaver & Burk (1934). This experiment gives K_m $2·8 \times 10^{-4}$M for galactose. At a galactose concentration of 40mM, the activity is 0·7 of the extrapolated maximum velocity. The velocity units are arbitrary.

potassium chloride (Fig. 2). Those fractions containing galactokinase activity were pooled to give fraction III.

(5) *Ammonium sulphate fractionation.* Solid ammonium sulphate was added to fraction III to bring the solution to 40% saturation. The precipitate was centrifuged off and discarded. The precipitate formed after bringing the solution to 60% saturation was collected and suspended in saturated ammonium sulphate (fraction IV). In suspension the enzyme loses about 20% of the enzyme activity in a week at 0–4°.

The purification procedure should not take longer than 24hr. if a reasonable yield of enzyme is required. The enzyme has been purified 250–500-fold and has a specific activity of 10–18 units/mg. of protein. A sample purification is shown in Table 1.

Properties of the purified galactokinase

Activation by thiols. Table 2 shows the activation of galactokinase by cysteine, glutathione, β-mercaptoethanol, ethanethiol and dithiothreitol. Activation was greatest with cysteine and least with ethanethiol. All the thiol reagents gave greater stimulation at a concentration of 5mM than at 1mM.

Effect of pH *on activity.* The pH optimum for galactokinase in the triethanolamine buffer is about 7·8. Below pH 7·2 and above pH 8·7 the enzyme activity was considerably lower than at the optimum pH. In this experiment 10 units each of pyruvate kinase and lactate dehydrogenase were used in the assays to prevent these enzymes from becoming rate-limiting at extreme pH.

Effect of temperature on activity. The enzyme activity was measured over the temperature range 20–40°. From these experiments the energy of activation for the reaction was calculated to be 12·4kcal./mole and the Q_{10} for ADP production about 2.

Substrate specificity. The K_m for galactose phosphorylation was between 1×10^{-4} and 3×10^{-4}M (Fig. 3). Inhibition by substrate occurs at

galactose concentrations greater than 2mM. Of the other substrates tested only 2-deoxygalactose and galactosamine showed any activity with the enzyme preparation. Both these compounds, which are different structurally from galactose at C-2, have $V_{max.}$ greater than galactose and Michaelis constants about 7–9 times as large (Table 3). No enzyme activity could be detected with mannose, fructose, glucose, 2-deoxyglucose or 6-deoxy-D-galactose and 6-deoxy-L-galactose at concentrations of 30mM, or galactose 1-phosphate, galactose 6-phosphate, lactose and *N*-acetylgalactosamine at concentrations of 3mM as substrates.

The effect of ATP concentration at equimolar concentrations of magnesium chloride is shown in Fig. 4. From this Lineweaver–Burk plot the K_m for MgATP was calculated to be $1{\cdot}9 \times 10^{-4}$M.

Product of galactokinase reaction. To a galactokinase assay mixture as described in the Materials and Methods section but containing 0·45μmole of [1-^{14}C]galactose with no additional galactose, there was added 0·1 unit of purified galactokinase. The extinction was followed until the reaction had almost stopped, to measure the ADP formed, and the assay mixture added to 30μmoles of galactose 1-phosphate and 20μmoles of galactose 6-phosphate and immediately placed on a column of Amberlite CG-400 (100–200 mesh) prepared in the borate form with bed dimensions 1cm.^{2} × 35cm. as described by Diedrich & Anderson (1961). The column was eluted with 900ml. of 0·1M-sodium tetraborate, pH 9·3, followed by 250ml. of a linear pH gradient to 0·1M-sodium tetraborate adjusted to pH 8·5 with 2N-sulphuric acid, and finally with 300ml. of this solution at pH 8·5 (Diedrich & Anderson, 1961). Carbohydrate was determined by the cysteine–sulphuric acid method as described by Diedrich & Anderson (1961). This system separates galactose 1-phosphate from galactose, galactose 6-phosphate, glucose 1-phosphate, glucose 6-phosphate and fructose 6-phosphate. All the radioactivity originating in [^{14}C]galactose was found in galactose 1-phosphate (95%, equivalent to 0·43μmole) and in remaining galactose (Fig. 5).

Stoicheiometry of the galactokinase reaction. Different amounts (0·18, 0·22 and 0·36μmole) of galactose were added to assay mixtures together with 0·1 unit of galactokinase and the reactions followed spectrophotometrically until completion. As shown in Table 4, 1μmole of ADP was formed

Table 3. *Substrate specificity of galactokinase*

Enzyme activity was determined as described in the Materials and Methods section. The rates of galactokinase action on galactose, 2-deoxygalactose and galactosamine are compared at the maximum velocity determined by extrapolation of Lineweaver–Burk plots. This extrapolation was necessary because galactokinase shows substrate inhibition at high galactose concentrations.

	Relative rate	K_m (mM)
Galactose	100	0·28
2-Deoxygalactose	128	2·4
Galactosamine	124	1·9

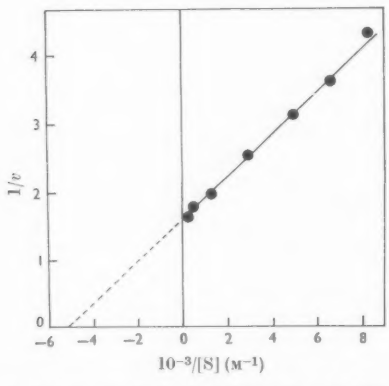

Fig. 4. Lineweaver–Burk plot of the effect of ATP concentration on galactokinase. This experiment gives K_m $1{\cdot}9 \times 10^{-4}$M for MgATP. The assay conditions are identical to those described in the Materials and Methods section except that the concentration of ATP and $MgCl_2$ were varied at a molar ratio of 1. The velocity units are arbitrary.

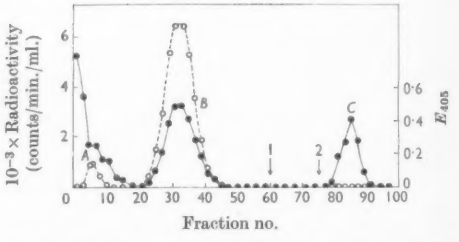

Fig. 5. Identification of galactose 1-phosphate as the product of the enzyme reaction. [1-^{14}C]Galactose was incubated in an assay system as described under 'Properties of the purified galactokinase'. After the reaction was nearly completed the reaction mixture was added to 30μmoles of galactose 1-phosphate and 20μmoles of galactose 6-phosphate and chromatographed on a column of Amberlite CG-400 (borate form). The column was eluted and the radioactivity (○) and carbohydrate (E_{405}, ●) were detected as described in the text. The pH gradient was started at arrow 1 and completed at arrow 2. Peak *A* is [^{14}C]galactose, *B* is galactose 1-phosphate and the ^{14}C-labelled product, and *C* is galactose 6-phosphate. Each fraction had a volume of 15ml.

Table 4. *Stoicheiometry of ADP formation*

In Expts. 1, 2 and 3, 0·18, 0·22 and 0·36 μmole of galactose, and in Expt. 4, 0·45 μmole of [1-^{14}C]galactose, were added to the assay system together with 0·1 unit of purified galactokinase. The amount of ADP formed was calculated from the extinction change at 340 mμ. The galactose 1-phosphate produced and the galactose remaining in Expt. 4 were determined by liquid-scintillation assays of the radioactivity eluted from a column of Amberlite CG-400 (see Fig. 5).

Expt. no.	Galactose added (μmole)	ADP formed (μmole)	Galactose 1-phosphate formed (μmole)	ADP/galactose ratio
1	0·18	0·19	—	1·06
2	0·22	0·21	—	0·95
3	0·36	0·37	—	1·03
4	0·45	0·43	0·43	1·00

for each μmole of galactose added. In addition, from the preceding experiment in which the phosphorylated product was found to be galactose 1-phosphate, 0·43 μmole of galactose 1-phosphate and 0·43 μmole of ADP were formed from 0·45 μmole of galactose and excess of ATP (Table 4).

DISCUSSION

The results in this paper with pig-liver galactokinase confirm and extend the work of Cardini & Leloir (1953) on crude preparations of galactokinase from rat liver. Although no evidence has been presented that the galactokinase is pure, the substrate-specificity studies and the omission of either pyruvate kinase or lactate dehydrogenase from the assay show that the preparation does not contain hexokinase, glucokinase, fructokinase, lactate dehydrogenase or pyruvate kinase, all of which are present in the liver extract.

The specific activity of galactokinase purified from liver (10–18 units/mg. of protein) is similar to the preparation from *E. coli* (14 units/mg.; Sherman & Adler, 1963) and greater than that purified from yeast by Heinrich (1964) or Howard & Heinrich (1965), namely 2·5–5 units/mg. Most of the properties of galactokinase purified from the three sources are similar. The pH optima, the Michaelis constant for ATP and the protection or stimulation by thiols are all alike. However, the substrate specificities of the preparations do differ. The Michaelis constant for galactose for the liver enzyme (1×10^{-4}–3×10^{-4} M) is lower than has been found for the *E. coli* enzyme (7×10^{-4} M; Sherman & Adler, 1963) or for the yeast enzyme ($1{\cdot}1 \times 10^{-3}$ M; Heinrich, 1964). Although the liver enzyme reacts with both galactosamine and 2-deoxygalactose besides galactose, both of these compounds have only marginal activity with the yeast enzyme (Heinrich, 1964).

As liver galactokinase is eluted from Sephadex G-100 at approximately the same volume as haemoglobin (as determined by extinction at 412 mμ; see Fig. 2*a*), the molecular weight of galactokinase would be approx. 60000. This is larger than the reported value of 20000 determined by sedimentation methods for the *E. coli* enzyme (Sherman & Adler, 1963).

After this research had been completed, some studies on the development and adaptive characteristics of galactokinase in rat liver were published by Cuatrecasas & Segal (1965). These authors found that galactokinase occurs in all tissues tested, but it had highest activity in liver from young rats. They also presented some kinetic evidence for the occurrence of two galactokinase enzymes in rat liver. In my purification procedure, no evidence for heterogeneity of galactokinase on Sephadex G-100, Sephadex G-200 or DEAE-cellulose chromatography was obtained. This does not eliminate the possibility that two galactokinase enzymes are present, as the proteins may have similar molecular weights and behaviour towards ion-exchange resins, or may not be present together in liver of the pig at the age chosen for the purification.

As young animals obtain galactose from lactose in milk, it is not surprising to find that the liver at this stage contains more galactokinase than in the adult. This enzyme may limit the rate of galactose metabolism as the liver from young animals have higher rates of galactose oxidation, uptake (Segal, Roth & Bertoli, 1963) and conversion into glycogen (Ballard & Oliver, 1964, 1965) than adult liver.

The author is indebted to Dr Sidney Weinhouse for many helpful discussions during these experiments. Financial assistance from the Commonwealth Scientific and Industrial Research Organisation of Australia, from U.S. National Institutes of Health Grants AM5487 and CA7174, and from The American Cancer Society Grant P202 is gratefully acknowledged.

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